

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

THIS PAGE BLANK (USPTO)

06-23-04

IFW

Express Mail Label No.

Dated: _____

Docket No.: 04305/100M237-US1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



Patent Application of:
Jens Holm et al.

Application No.: 10/698,855

Confirmation No.: 9333

Filed: October 31, 2003

Art Unit: N/A

For: RECOMBINANT PROTEIN VARIANTS

Examiner: Not Yet Assigned

CLAIM FOR PRIORITY AND SUBMISSION OF DOCUMENTS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant hereby claims priority under 35 U.S.C. 119 based on the following prior foreign application filed in the following foreign country on the date indicated:

Country	Application No.	Date
Denmark	PA 2002 01686	November 1, 2002

In support of this claim, a certified copy of the said original foreign application is filed herewith.

Dated: June 21, 2004

Respectfully submitted,

By Marc S. Gross
Marc S. Gross

Registration No.: 19,614
DARBY & DARBY P.C.
P.O. Box 5257
New York, New York 10150-5257
(212) 527-7700
(212) 753-6237 (Fax)
Attorneys/Agents For Applicant

THIS PAGE BLANK (USPTO)



Application No. (if known): 10/698,855

Attorney Docket No.: 04305/100M237-US1

Certificate of Express Mailing Under 37 CFR 1.10

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail, Airbill No. EV418264057 US in an envelope addressed to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

on June 21, 2004
Date

Patricia A. Rubio

Signature

PATRICIA A. RUBIO

Typed or printed name of person signing Certificate

Note: Each paper must have its own certificate of mailing, or this certificate must identify each submitted paper.

Claim for Priority and Submission of Documents (1 page)
A certified copy of one (1) priority document
Return Postcard

THIS PAGE BLANK (USPTO)



Kongeriget Danmark

Patent application No.: PA 2002 01686

Date of filing: 01 Nov 2002

Applicant:
(Name and address)
ALK-Abello A/S
Bøge Allé 6-8
DK-2970 Hørsholm
Denmark

Title: Rekombinante proteinvarianter.

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.



Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

26 May 2004

Pia Petersen



PATENT- OG VAREMÆRKESTYRELSEN

THIS PAGE BLANK (USPTO)

RECOMBINANT PROTEIN VARIANTS**FIELD OF THE INVENTION**

5

The present invention relates to immunotherapy, in particular the present invention relates to allergy vaccination strategies employing novel recombinant proteins.

10 Also, the invention relates to a composition comprising a mixture of the novel recombinant protein variants.

BACKGROUND OF THE INVENTION

15 Allergy is a major health problem in countries where Western lifestyle is adapted. Furthermore, the prevalence of allergic disease is increasing in these countries. Although allergy in general may not be considered a life-threatening disease, asthma annually causes a significant number of deaths. An exceptional prevalence of about 30% in teenagers conveys a substantial 20 loss in quality of life, working days and money, and warrants a classification among major health problems in the Western world.

Allergy is a complex disease. Many factors contribute to the sensitisation event. Among these is the susceptibility of the individual defined by an as yet 25 insufficiently understood interplay between several genes. Another important factor is allergen exposure above certain thresholds. Several environmental factors may be important in the sensitisation process including pollution, childhood infections, parasite infections, intestinal microorganisms, etc. Once an individual is sensitised and the allergic immune response established, the 30 presence of only minute amounts of allergen is efficiently translated into symptoms.

The natural course of allergic disease is usually accompanied by aggravation at two levels. Firstly, an progression of symptoms and disease severity, as

well as disease progression, for example from hay fever to asthma. Secondly, dissemination in offending allergens most often occurs resulting in allergic multi-reactivity. Chronic inflammation leads to a general weakening of the mucosal defense mechanisms resulting in unspecific irritation and

5 eventually destruction of the mucosal tissue. Infants may become sensitised primarily to foods, i.e. milk, resulting in eczema or gastrointestinal disorders; however, most often they outgrow these symptoms spontaneously. These infants are at risk of developing inhalation allergy later in their lives.

10 The most important allergen sources are found among the most prevalent particles of a certain size in the air we breathe. These sources are remarkably universal and include grass pollens and house dust mite faecal particles, which together are responsible for approximately 50% of all allergies. Of global importance are also animal danders, i.e. cat and dog

15 dander, other pollens, such as mugwort pollens, and micro-fungi, such as Alternaria. On a regional basis yet other pollens may dominate, such as birch pollen in Northern and Central Europe, ragweed in the Eastern and Central United States, and Japanese cedar pollen in Japan. Insects, i.e. bee and wasp venoms, and foods each account for approximately 2% of all allergies.

20 All IgE-mediated allergic responses are directed towards proteins. In recent years the characterization of allergen molecules has advanced considerably facilitated by the application of modern techniques within molecular and structural biology. These studies have failed to demonstrate the existence of

25 a common structural motif or a biological function shared by all allergens. It must be anticipated that any protein is a potential allergen, and that the most important allergens, i.e. the major allergens, are the most abundant proteins on airborne particles of a size facilitating their presentation to the immune system.

30 Allergy, i.e. type I hyper-sensitivity, is caused by an inappropriate immunological reaction to foreign non-pathogenic substances. Important clinical manifestations of allergy include asthma, hay fever, eczema, and gastrointestinal disorders. The allergic reaction is prompt and peaks within

20 minutes upon contact with the offending allergen. Furthermore, the allergic reaction is specific in the sense that a particular individual is sensitised to particular allergen(s), whereas the individual does not necessarily show an allergic reaction to other substances known to cause

5 allergic disease. The allergic phenotype is characterized by a pronounced inflammation of the mucosa of the target organ and by the presence of allergen specific antibody of the IgE class in the circulation and on the surface of mast-cells and basophils.

10 An allergic attack is initiated by the reaction of the foreign allergen with allergen specific IgE antibodies, when the antibodies are bound to high affinity IgE specific receptors on the surface of mast-cells and basophils. The mast-cells and basophils contain preformed mediators, i.e. histamine, tryptase, and other substances, which are released upon cross-linking of two

15 or more receptor-bound IgE antibodies. IgE antibodies are cross-linked by the simultaneous binding of one allergen molecule. It therefore follows that a foreign substance having only one antibody binding epitope does not initiate an allergic reaction. The cross-linking of receptor bound IgE on the surface of mast-cells also leads to release of signaling molecules responsible for the

20 attraction of eosinophils, allergen specific T-cells, and other types of cells to the site of the allergic response. These cells in interplay with allergen, IgE and effector cells, lead to a renewed flash of symptoms occurring 12-24 hours after allergen encounter.

25 Allergy disease management comprises diagnosis and treatment including prophylactic treatments. Diagnosis of allergy is concerned with the demonstration of allergen specific IgE and identification of the allergen source. In many cases a careful anamnesis may be sufficient for the diagnosis of allergy and for the identification of the offending allergen source

30 material. Most often, however, the diagnosis is supported by objective measures, such as skin prick test, blood test, or provocation test. The principle behind the blood test is the demonstration of specific IgE using an array of allergen sources. The therapeutic options fall in three major categories. The first opportunity is allergen avoidance or reduction of the

exposure. Whereas allergen avoidance is obvious e.g. in the case of food allergens, it may be difficult or expensive, as for house dust mite allergens, or it may be impossible, as for pollen allergens. The second and most widely used therapeutic option is the prescription of symptomatic drugs.

5 Symptomatic drugs are safe and efficient; however, they do not alter the natural cause of the disease, neither do they control the disease dissemination. The third therapeutic alternative is specific allergy vaccination that in most cases reduces or alleviates the allergic symptoms caused by the allergen in question.

10 Specific allergy vaccination is a causal treatment for allergic disease. It interferes with basic immunological mechanisms resulting in persistent improvement of the patients' immune status. Thus, the protective effect of specific allergy vaccination extends beyond the treatment period in contrast

15 to symptomatic drug treatment. Some patients receiving the treatment are cured, and in addition, most patients experience a relief in disease severity, or at least an arrest in disease aggravation. Thus, specific allergy vaccination has preventive effects reducing the risk of hay fever developing into asthma, and reducing the risk of developing new sensitivities.

20 Specific allergy vaccination is, in spite of its virtues, not in widespread use, primarily for two reasons. One reason is the inconveniences associated with the traditional vaccination programme that comprises repeated vaccinations over a several months. The other reason is, more importantly, the risk of

25 allergic side reactions. Ordinary vaccinations against infectious agents are efficiently performed using a single or a few high dose immunizations. This strategy, however, cannot be used for allergy vaccination since a pathological immune response is already ongoing.

30 Specific allergy vaccination is therefore carried out using multiple immunizations applied over an extended time period. The course is divided in two phases, the up dosing and the maintenance phase. In the up dosing phase increasing doses are applied, typically over a 16 week period, starting with minute doses. When the recommended maintenance dose is reached,

this dose is applied for the maintenance phase, typically with injections every six weeks. Following each injection the patient must remain under medical attendance for 30 minutes due to the risk of anaphylactic side reactions, which in principle could be life-threatening. In addition, the clinic should be 5 equipped to support emergency treatment. There is no doubt that eliminating the risk for allergic side reactions inherent in the vaccine would facilitate a more general use, possibly even enabling self vaccination at home.

The immunological mechanism underlying successful allergy vaccination is 10 not known in detail. A specific immune response, such as the production of antibodies against a particular pathogen, is known as an adaptive immune response. This response can be distinguished from the innate immune response, which is an unspecific reaction towards pathogens. An allergy vaccine is bound to address the adaptive immune response, which includes 15 cells and molecules with antigen specificity, such as T-cells and the antibody producing B-cells. B-cells cannot mature into antibody producing cells without help from T-cells of the corresponding specificity. T-cells that participate in the stimulation of allergic immune responses are primarily of the Th2 type. Establishment of a new balance between Th1 and Th2 cells has been 20 proposed to be beneficial and central to the immunological mechanism of specific allergy vaccination. Whether this is brought about by a reduction in Th2 cells, a shift from Th2 to Th1 cells, or an up-regulation of Th1 cells is controversial. Recently, regulatory T-cells have been proposed to be 25 important for the mechanism of allergy vaccination. According to this model regulatory T-cells, i.e. Th3 or Tr1 cells, down-regulate both Th1 and Th2 cells of the corresponding antigen specificity. In spite of these ambiguities it is generally believed that an active vaccine must have the capacity to stimulate allergen specific T-cells, preferably TH1 cells.

30 With respect to allergen specific antibodies during the course of specific allergy vaccination an important observation is that in spite of clinical improvement there seem to be no corresponding change in IgE. Allergen specific IgE may provisionally rise early in treatment, but then falls back to pre-treatment levels during the treatment and may subsequently show a

gradual decrease. Clinical improvement, on the other hand, is steadily increasing during the entire treatment period.

Another important observation is a large increase in allergen specific IgG 5 early in the treatment period. Allergen specific IgG typically increases to concentrations hundreds or thousands times that of IgE. It has been proposed that allergen specific IgG is important for the immunological mechanism of specific allergy vaccination, since it can interfere or 'block' the interaction between IgE and the allergen. According to this model it is likely 10 that IgG needs to undergo affinity maturation by repeated immunizations before it can efficiently compete with IgE for binding to the allergen explaining the steady increase in clinical improvement observed during treatment.

The human immune system has two different ways of specific molecular 15 recognition: via the T-cell receptor present on the surface of T-cells, and via the antibodies, which may be bound to the surface of the B-cells producing them, they may be free in solution, or they may be bound to specific receptors on the surface of a variety of cells in the immune system. Recognition via the T-cell receptor requires that the antigen is internalized 20 and digested, and a fragment of the antigen is presented on the cell surface in complex with major histocompatibility complex (MHC), by an antigen presenting cell. The entire complex is recognized by the T-cell receptor. The antigen presenting cell can be a professional antigen presenting cell, i.e. a macrophage, dendritic cell or a B-cell. Macrophages and dendritic cells 25 ingest antigen by phagocytosis; whereas B-cells, capture antigen via surface bound antibody for subsequent internalisation and presentation. A T-cell epitope is a polypeptide fragment of 15-20 amino acids.

The B-cell epitope, i.e. antibody binding epitope, is different from the linear T- 30 cell epitopes. Although antibodies that are raised in experimental animals by immunization in Freunds complete adjuvant may bind to linear polypeptide fragments, i.e. sequential epitopes, such antibodies are rarely raised during the natural response to allergens. Antibodies bind to a section of the surface of the correctly folded antigen molecule. IgE epitopes of naturally occurring

allergens are thus envisaged to consist of a number of surface exposed amino acids in a confined surface area that are supported by the backbone and the overall three-dimensional structure of the allergen. Such "epitope amino acids" are almost always found as either single amino acids or in 5 clusters consisting of a few amino acids distributed over a large portion of the primary sequence of the allergen "brought together" on the surface upon folding of the molecule. The antibody binding epitope extends typically over approximately 800-900 Å², but an even larger area is "masked" by the binding of the antibody. This means that upon binding of an antibody to an 10 epitope, an area of up to 2000 Å² becomes inaccessible to binding of other antibody molecules.

The affinity of the interaction between the antibody and the antigen is not only dependent on the attracting electrostatic forces, i.e. van der Waal's 15 interactions, hydrogen bonding, and ion bridges, but also on the entropy gained by the almost complete expulsion of water molecules from the interface. Thus, the fit between the contours of the two molecular surfaces may be said to be an important parameter in defining the binding strength of the interaction. It follows that denaturation, i.e. unfolding, of the antigen (or 20 allergen) usually destroys the antibody binding epitope, and binding of antibody will no longer be feasible in a physiological concentration range. It follows also, that if an amino acid in the epitope is substituted with another amino acid it will result in a reduction of the affinity of the interaction. The extent of the reduced antibody binding affinity depends on both the specific 25 substituted amino acid and the antibody in question. It has e.g. been shown that one single amino acid substitution in an antigen can affect the interaction between antigen and antibody by a factor one thousand.

Attempts to improve vaccines for specific allergy vaccination by chemical 30 modification have been performed for over 30 years and include multifarious approaches with one overall objective: to improve safety while not compromising efficacy; or in other words, to reduce IgE binding while not compromising immunogenicity.

Initial approaches included complete denaturation of the allergen. However, human trials have failed to show efficacy, which could be due to the non-efficient generation of a protective immune response. For the purpose of addressing T-cells in the allergic immune response, medium sized synthetic peptides (50-60 amino acids in length) were tested in human trials. Although the peptides showed some effect in very high doses, the trials were terminated due to the appearance of irregular side reactions. From these trials it seems that efficacy is compromised in the absence of antibody binding epitopes in the vaccine.

10

Other early attempts included chemical modification of allergen extracts either by acetylation (epitope masking), conjugation to large polymers (polyethyleneglycol), or polymerization into large so-called 'allergoids' by formaldehyde or glutaraldehyde. The former two concepts never entered human trials, whereas the 'allergoids' are currently in routine clinical use, however, without the benefit of improved safety. The reason for this is probably that chemical cross-linking randomly destroys some of the epitopes, which necessitates the use of higher doses for optimal efficacy, thereby increasing the level of allergic side effects approximately to the level observed using conventional allergy vaccines.

20

Approaches to improve allergy vaccines include various strategies to disrupt the three-dimensional structure of the allergen vaccine molecules. Among these are substitutions of cysteine residues taking part in disulphide bridging. Another strategy is to assemble several naturally occurring amino acid substitutions into one molecule by site directed mutagenesis. A common characteristic of these strategies is to disrupt the tertiary structure of the vaccine, or at least not to consider preservation of the three-dimensional structure important.

25

Another commonly used approach has been to produce recombinant mutant variants of naturally occurring allergens. The mutations are usually selected so as to reduce IgE affinity of the mutant protein.

WO 99/47608 discloses the introduction of artificial amino acid substitutions into defined critical positions of the naturally occurring allergen while retaining the α -carbon backbone tertiary structure of the allergen.

5 WO 02/40676 discloses the introduction of at least 4 primary artificial amino acid substitutions into defined critical positions while retaining the α -carbon backbone tertiary structure of the allergen, wherein each primary mutation is spaced from another primary mutation by at least 15 Å and wherein at least one circular surface region with an area of 800 Å² comprises no mutation.

10 J. Biomol. Struc. Dynamics (2000), vol. 17 pages 821-828 (Liang et al.) discloses transferring of a biological function of one protein to another by grafting the functional part of the protein to another appropriate scaffold protein. As a test system, the binding epitope of barstar, the inhibitor of 15 barnase, is grafted onto a smaller molecule.

Protein Science (1994), vol. 3, p. 2351-2357 (Jin & Wells) discloses use of antigen-antibody complexes for studying protein-protein interactions. The binding of human growth hormone hGH to a monoclonal antibody (MAb 3) 20 was studied. Five amino acids of hGH essential for the binding to MAb 3 was grafted onto a non-binding homologue of hGH, human placental lactogen (hPL), which has 86 % sequence identity with hGH. Also, two additional framework mutations were introduced. The grafted hPL mutant bound to MAb 3 as well as hGH. Attempts to graft the epitope onto a scaffold protein having 25 a homology with hGH of 23 % were not successful.

J. Immunol (2001), vol. 166, p. 6057-6065 (King et al.) discloses hybrid insect venom allergens. The hybrids consist of an insertion of a small portion of the guest allergen of interest, Ves v 5, into a homologous host protein, Pol a 5. 30 Ves v 5 and Pol a 5 have a sequence identity of 59 % and a low degree of antigenic cross-reactivity. Hybrids are formed by inserting one portion of about 7 to about 50 amino acids into the allergen. The hybrids are useful for the mapping of B-cell epitope-containing regions of proteins and for use as immunotherapeutic reagents in man. This approach for generating allergen

hybrids is however not appropriate for use in allergy vaccination strategies for the following reasons. Firstly, insertion of a peptide fragment from an insect allergen into an insect scaffold protein will in many cases lead to destabilization of the three-dimensional structure of the molecule. Unstable molecules are not suitable for use in vaccination. Secondly, as epitopes almost never consist of a single linear peptide fragment of the allergen, this approach is not suitable for "grafting" three-dimensional epitopes from allergens to scaffold proteins.

10 **OBJECT OF THE INVENTION**

The object of the invention is to provide new protein variants of naturally occurring allergens, said protein variants having properties useful in allergy vaccination. Accordingly, it is the aim of the current invention to generate protein variants of scaffold proteins for use as vaccine components, which resemble natural allergens with respect to the overall protein folding pattern and the contours of the surface topography, while at the same time having surface contours that have low or reduced IgE binding capability compared to the allergen. The purpose is to generate surface contours of the scaffold protein with allergen specific IgE cross-reactivity, in order to enable stimulation of immune responses that will generate protective IgG antibodies with the ability to block IgE binding to the natural allergen and thereby alleviate or cure allergy symptoms.

25 Previous approaches for obtaining protein variants with improved performance in e.g. allergy vaccines have mainly focused on modification of the naturally occurring allergen in order to reduce IgE binding affinity. A major disadvantage of this approach is that by introducing an increasing number of mutations into the allergen, the risk of destabilizing the three-dimensional structure of the molecule is greatly increased and unstable molecules are certainly not attractive to use as therapeutic vaccine candidates.

30 The idea behind the present invention is to select a naturally occurring "scaffold protein" with sequence homologies to the natural allergen. The

scaffold protein has a folding pattern that resembles that of the natural allergen while at the same time displaying substantially reduced antibody cross-reactivity. In this way it is ensured that the "starting material" is a stable molecule with correct three-dimensional folding pattern and with little or no cross-reactivity with allergen specific IgE antibodies. Introduction of mutations in the scaffold protein, introducing or modulating or eliminating existing antibody binding surface contours or epitopes homologous to structures of the allergen, results in creation of stable protein variants, capable of raising a protective immune response and with a lowered risk of inducing side-effects, since the mutated scaffold protein variant exhibits a lower antibody reactivity compared to the natural allergen

The invention is based on the recognition that it is possible to model partly or fully an epitope of an allergen on a scaffold protein, by substituting amino acids of the portion of the scaffold protein surface area corresponding to the epitope by amino acids located in the corresponding position in the primary amino acid sequence of the allergen. In particular, the invention is based on the recognition that even though most epitopes are discontinuous, it is nevertheless possible to create such epitopes fully or partly by selective and specific mutation of key surface amino acids of the epitopes situated at different positions of the primary amino acid sequence. Furthermore, the invention is based on the discovery that such mutated scaffold protein variants may be used in lieu of the native allergens for therapeutic and diagnostic purposes, and that such mutated scaffold proteins have modified properties for binding of antibodies and hence i.e. have the advantage of a reduced risk of inducing anaphylactic reactions when used as an allergy vaccine component.

A more efficient generation of a protective immune response can be achieved by using a mixture of different protein variants, thereby presenting the immune system to several different epitope areas, preferably without presenting more than one epitope area at the same molecule.

One aspect of the present invention comprises introduction of primary mutations in a defined, coherent surface area of up to about 5000 Å² in order to establish a surface area that mimics an epitope on the natural allergen. The surface area to be mutated is restricted to 5000 Å² for the purpose of allowing only a single antibody molecule to bind at a time thereby avoiding the risk of effector cell triggering in a vaccination scenario. Preferably, the scaffold protein has little or no measurable cross-reactivity with allergen specific IgE antibodies and the resulting mutated allergen variant thus preferably only has the ability to bind one antibody molecule.

Another aim of the present invention comprises introduction of mutations for the purpose of establishing several surface areas that mimick allergen epitopes. Also, mutations may be introduced in order to reduce affinity of other surface areas with allergen specific IgE cross-reactivity. Several antibody molecules will thus in theory be able to bind to the mutated protein variant in question, but with greatly reduced affinities compared to binding of these antibodies to the natural allergen. The affinities of the IgE interactions should be reduced to a level limiting or abolish the risk of triggering effector cell degranulation, while at the same time retaining the capacity to induce formation of protective antibodies reactive with the allergen in question.

DESCRIPTION OF THE DRAWINGS

Figure 1

Mutation by site directed mutagenesis. Punctured lines represent DNA sequences. Numbers in parentheses above lines represents sense oligonucleotide primers: (1), (3), (5), (7), (9), (11). Numbers in parentheses below lines represents anti-sense oligonucleotide primers: (2), (4), (6), (8), (10), (12). Notation X (position (pos)) Y represents mutations at different positions. (1) Represents the sense oligonucleotide primer accommodating the protein N-terminus. (12) Represents the anti-sense oligonucleotide primer accommodating the protein C-terminus.

Figure 2

5 Circular dichroism (CD) spectroscopy. CD spectra of rMal d 1 (2620) (■), rBet v 1.2801 (●), mutated rMal d 1 (2762) (◊) and mutated rMal d 1 (2781)(△).

10

Figure 3A and 3B

Inhibition of the binding of biotinylated rBet v 1.2801 to pooled IgE serum from birch allergic patients by rBet v 1.2801 (●), rMal d 1 (2620) [■] or mutated rMal d 1 (2760) [□] with three secondary mutations. A = serum pool 10 0202/148. B=serum pool 11-97.

15

Figure 4

Heat-denaturing of rMal d 1 (2620). Overlay of circular dichroism (CD) spectra obtained at 15°C to 90°C are shown. Native folded (15°C) rMal d 1 (2620) has negative and positive amplitudes at 214-216 nm and 192-194 nm, respectively. Heat denatured (90°C) rMal d 1 (2620) protein preparations have negative amplitudes at 204 nm and considerably less negative and positive values at 214-216 nm and 192-194 nm, respectively, compared to native folded allergen. The protein preparation heated to 90 °C was cooled 20 down for an additional recording at 15 °C (○) which is overlapping with the spectrum of native folded protein preparations. Furthermore, the CD-spectra of rMal d 1 (2620) recorded at 70°C are only slightly altered compared to the spectrum recorded at 15C.

25

Figure 5A, B and C

three-dimensional-structures of rMal d 1 (2620) (A,B) and rMal d 1 (2760) (C). Molecular surfaces are shown. Grey surface areas show Bet v 1.2801 specific amino acid residues on Mal d 1. White surface areas show Mal d 1 (2620) specific amino acid residues. A and C = front view. B = back view. 30 One large birch-apple antibody cross-reactive surface area is framed in black in figure A. In figure C three secondary mutations N28T, K32Q, E45S in rMal d 1 (2760) are shown in black. Two of the secondary mutations N28T, K32Q affect both the topography as well as the amino acid charge distribution in the large birch-apple antibody cross-reactive surface area.

Figure 6A, B and C

three-dimensional-structures of rMal d 1 (2620) (A), rMal d 1 (2781) (B) and rBet v 1.2801 (C). Molecular surfaces (front views) are shown. Grey surface areas show Bet v 1.2801 specific amino acid residues. White surface areas show Mal d 1 (2620) specific amino acid residues. In figure B, the six primary mutations introduced in rMal d 1 (2781) are shown in black. Figure C shows the same amino acid residues in black in Bet v 1.2801. Comparing figure A, B and C show that the six primary mutations in rMal d 1 (2781) introduce Bet v 1 specific surface structures onto one surface area on rMal d 1.

10

Figure 7A, B and C

three-dimensional-structures of rMal d 1 (2620) (A), rMal d 1 (2762) (B) and rBet v 1.2801 (C). Molecular surfaces (back views) are shown. Grey surface areas show Bet v 1.2801 specific amino acid residues. White surface areas show Mal d 1 (2620) specific amino acid residues. In figure B the eight primary mutations introduced in rMal d 1 (2762) are shown in black color. Figure C shows the same amino acid residues in black in Bet v 1.2801. Comparing figure A, B and C show that the eight primary mutations in rMal d 1 (2762) introduce Bet v 1 specific surface structures onto one surface area on rMal d 1.

15

Figure 8 A and B

A. Inhibition of the binding of biotinylated rBet v 1.2801 to pooled IgE serum from birch allergic patients by rBet v 1.2801 (●), rMal d 1 (2620) (■) or mutated rMal d 1 (2762) (◊) or (2781) (△) with 8 and 6 primary mutations, respectively.

20

B. Histamine release of rMal d1 and mutated rMal d 1 in comparison to rBet v1.2802. rBet v 1.2801 (●), rMal d 1 (2620) (■) or mutated rMal d 1 (2762) (◊) or (2781) (△) with 8 and 6 primary mutations, respectively.

Figure 9

The graphics show results from IgE-inhibition experiments (right) and histamine release experiments (left) with serum from 5 individual patients. Right side: inhibition curves of binding of human serum IgE to rBet v 1.2801 (•) for rMal d 1 (2620) (■) and mutated rMal d 1 2760 (□). Left side: histamine release of rMal d 1 and mutated rMal d 1 in comparison to rBet v 1.2801

Figure 10A and B.

10 Schematic illustration of a scaffold protein and a scaffold variant derived from the scaffold protein by introducing primary and secondary mutations.

Figure 11 A, B and C

15 T-cell proliferative response to rMAL d 1 allergens of three individual patients: MCDS12 (A), GUA (B) and AHB21 (C).

Figure 12

T-cell stimulation index of the three patients in Figure 11 (A, B, C).

Figure 13

20 T-cell proliferative response of Bet v 1 specific T-cell line.

Figure 14

Cytokine response to Mal d 1 allergens in PBL cultures.

25

Figure 15

Cytokine response to Mal d 1 allergens in Bet v1-specific T-cell lines.

Figure 16

30 Grafting Bet v 1 specific surface area I. Amino acid alignment of Dau c 1 and Bet v 1. Position no. above sequences refers to Dau c 1 (T14325). Positions no. below sequences refers to Bet v 1 (z80104). Black background shows positions in the polypeptide sequences having identical amino acid residues. Grey background shows positions in the polypeptide sequences having

homologous amino acid residues. Amino acid positions that in example 1A are targeted for introduction of secondary mutations are indicated with triple bars on black background and amino acid positions for introduction of primary mutations are indicated with black arrows.

5

Figure 17

Grafting Bet v 1 specific surface area II. Amino acid alignment of Dau c 1 and Bet v 1. Position no. above sequences refers to Dau c 1 (T14325). Positions no. below sequences refers to Bet v 1 (z80104). Black background shows 10 positions in the polypeptide sequences having identical amino acid residues. Grey background shows positions in the polypeptide sequences having homologous amino acid residues. Amino acid positions that in example 1B are targeted for introduction of secondary mutations are indicated with triple bars on black background and amino acid positions for introduction of 15 primary mutations are indicated with black arrows.

Figure 18

Amino acid alignment of Lep d 2 and Der p 2. Position no. above sequences refers to Lep d 2 (S66499). Positions no. below sequences refers to Der p 2 (P49278). Black background shows positions in the polypeptide sequences having identical amino acid residues. Grey background shows positions in the polypeptide sequences having homologous amino acid residues. Amino acid positions that in example 2A are targeted for introduction of primary 20 mutations are indicated with white arrows on black background. Amino acid positions that in example 2B are targeted for introduction of primary 25 mutations are indicated with black arrows on white background. Amino acid positions suggested for introduction of secondary mutations in either example 2A or 2B are indicated with triple bars on black background

30

Figure 19

View of amino acid residue positions on Lep d 2: K6, S22, R30, K76, K81, V114 that are selected in example 2A as possible target positions for the introduction of secondary mutations. ABCD show molecular surfaces of Lep d 2 (accession no S66499) modeled at

<http://www.expasy.org/swissmod/SWISS-MODEL.html> using pdb entry 1ktj as template. A= front view, B= Turn 1 (front view turned 90° out of plane around the horizontal axes), C= Turn 2 (front view turned 180° out of plane around the horizontal axes), D= Turn 3 (front view turned 270° out of plane around the horizontal axes). **Grey color** shows α -carbon backbone atoms and side chain atoms on amino acid residues that are identical or homologous to Der p 2 specific amino acid residues in corresponding amino acid positions. **White color** shows amino acid residues that are specific for Lep d 2 (accession no S66499). **Black color** shows amino acid residues on Lep d 2 that in example 2A are selected as possible targets for the introduction of secondary mutations.

Figure 20

View of amino acid residue positions on Lep d 2: D17, S19, Q32, K33, T35, N88, T92, A95, that are selected in example 2A as possible target positions for the introduction of primary mutations. ABCD show molecular surfaces of Lep d 2 (accession no S66499) modeled at <http://www.expasy.org/swissmod/SWISS-MODEL.html> using pdb entry 1ktj as template. A= front view, B= Turn 1 (front view turned 90° out of plane around the horizontal axes), C= Turn 2 (front view turned 180° out of plane around the horizontal axes), D= Turn 3 (front view turned 270° out of plane around the horizontal axes). **Grey color** shows α -carbon backbone atoms and side chain atoms on amino acid residues that are identical or homologous to Der p 2 specific amino acid residues in corresponding amino acid positions on Der p 2 (pdb entry 1ktj). **White color** shows amino acid residues that are specific for Lep d 2 (accession no s66499). **Black color** shows amino acid residues on Lep d 2 that in example 2A are selected as possible targets for the introduction of primary mutations.

Figure 21

Amino acid alignment of Mal d 1 (2620) and Bet v 1.2801. Position no. above sequences refers to Mal d 1 (accession no. AJ488060). Positions no. below sequences refers to Bet v 1 (accession no. Z80104). Black background shows positions in the polypeptide sequences having identical amino acid

residues. Grey background shows positions in the polypeptide sequences having homologous amino acid residues. Amino acid positions that in example 3 are targeted for introduction of secondary mutations are indicated with triple bars on black background and amino acid positions for introduction of primary mutations are indicated with black arrows.

Figure 22

Amino acid alignment of Gly d 2 and Der p 2. Position no. above sequences refers to Gly d 2 (AJ272216). Positions no. below sequences refers to Der p 2 (P49278). Black background shows positions in the polypeptide sequences having identical amino acid residues. Grey background shows positions in the polypeptide sequences having homologous amino acid residues. Amino acid positions that in example 4 are targeted for introduction of secondary mutations are indicated with triple bars on black background and amino acid positions for introduction of primary mutations are indicated with black arrows.

Figure 23

View of amino acid residue positions on Gly d 2 that are selected in example 4 as possible target positions for the introduction of secondary mutations. ABCD show molecular surfaces of Gly d 2 (accession no. AJ272216) modeled at <http://www.expasy.org/swissmod/SWISS-MODEL.html> using pdb entry 1ktj as template. A= front view, B= Turn 1 (front view turned 90° out of plane around the horizontal axes), C= Turn 2 (front view turned 180° out of plane around the horizontal axes), D= Turn 3 (front view turned 270° out of plane around the horizontal axes). **Grey color** shows α -carbon backbone atoms and side chain atoms on amino acid residues that are identical or homologous to Der p 2 specific amino acid residues in corresponding amino acid positions on Der p 2 (pdb entry 1ktj). **White color** shows amino acid residues that are specific for Gly d 2 (accession no. AJ272216). **Black color** shows amino acid residues on Gly d 2 that in example 4 are selected as possible targets for the introduction of secondary mutations.

Figure 24

View of amino acid residue positions on Gly d 2 that are selected in example 4 as possible target positions for the introduction of primary mutations. ABCD show molecular surfaces of Gly d 2 (accession no. AJ272216) modeled at 5 <http://www.expasy.org/swissmod/SWISS-MODEL.html> using pdb entry 1ktj as template. A= front view, B= Turn 1 (front view turned 90° out of plane around the horizontal axes), C= Turn 2 (front view turned 180° out of plane around the horizontal axes), D= Turn 3 (front view turned 270° out of plane around the horizontal axes). Grey color shows α -carbon backbone atoms 10 and side chain atoms on amino acid residues that are identical or homologous to Der p 2 specific amino acid residues in corresponding amino acid positions on Der p 2 (pdb entry 1ktj). White color show amino acid residues that are specific for Gly d 2 (accession no. AJ272216). Black color 15 shows amino acid residues on Gly d 2 that in example 4 are selected as possible targets for the introduction of primary mutations.

Figure 25

Composition in secondary structure elements of Mal d 1 (2620), Mal d 1 20 (2762), Mal d 1 (2781) and Bet v 1.2801, obtained by the deconvolution of their CD spectra. The deconvolution sets of Mal d 1 (2762), Mal d 1 (2781) and Bet v 1.2801 fall within the acceptance boundaries determined for the reference protein, Mal d 1 (2620) ($\pm 15\%$), and therefore these four proteins may be considered to be structurally (secondary structure) similar.

25 SUMMARY OF THE INVENTION

The present invention relates to a recombinant protein variant with the ability 30 to induce a protective immune response to a naturally occurring allergen, wherein the protein variant

- is a variant of a scaffold protein, said scaffold protein having a three-dimensional folding pattern that is structurally similar to that of the naturally occurring allergen,

- compared to the scaffold protein, comprises two or more primary mutations spaced by at least one non-mutated amino acid residue, each primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to the corresponding amino acid residue or residues in the naturally occurring allergen, and
- has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.

10 The present invention furthermore relates to the use of one or more protein variants for preparing a pharmaceutical composition for treatment of allergic individuals.

15 The present invention also relates to DNA sequences encoding protein variants according to the present invention as well as to vectors and host-cells hosting such DNA sequences and methods of producing recombinant proteins.

Also, the present invention relates to diagnostic assays.

20 DETAILED DESCRIPTION OF THE INVENTION

The idea of the present invention is to use a "scaffold protein" with a similar three-dimensional folding pattern but with little or no cross-reactivity with a naturally occurring allergen as the basis for creating novel allergen mutants.

25 This scaffold protein can be used for creating novel recombinant protein variants that possess surface areas with and without cross-reactivity with the allergen specific antibodies. There are several important advantages by using the scaffold protein as the basis for creating allergen vaccine candidates. It is usually required to perform a large number of mutations of an allergen in order to lower its allergen specific IgE reactivity and thereby the risk of inducing adverse effects during vaccination. Also, as a large number of mutations tend to destabilize the protein, these mutated allergens are often not suitable for use as vaccine candidates. The idea behind the

invention is thus that a relatively small number of mutations are generally required in order to partly or fully establish allergen specific IgE recognizing contours on the surface of an appropriate scaffold protein. Such molecules have the potential of inducing new protective immune responses that can 5 compete with IgE binding upon allergen exposure leading to a reduced risk of inducing IgE-mediated allergic responses.

One aspect of the invention is to generate a mutated protein variant with one allergen homologous surface area. A preferred scaffold protein according to 10 this aspect is a scaffold with little or no IgE cross-reactivity as a starting point thereby minimizing the need for introduction of secondary mutations. A scaffold with some cross-reactivity to allergen specific antibodies may also be suitable. Introduction of one or more secondary mutations in such a scaffold protein may limit or eliminate potential cross-reactivity. Cross-reactivity of 15 scaffold proteins and scaffold protein variants with the naturally occurring allergen can be tested by RAST inhibition using a panel of sera from patients allergic to the allergen in question (Nolte et al (1987) Allergy, 42:366-373). Devoid in antibody binding means a reduction in antibody affinity by a factor of 10^3 , more preferably 10^4 , most preferably 10^5 or more.

20 Comparison of structural similarities between the scaffold protein and the allergen in question includes assessment of three-dimensional structures and/or amino acid sequence. Folding pattern similarity is preferably assessed by comparison of scaffold and allergen three-dimensional structures. In 25 absence of a known three-dimensional structure of the scaffold protein and/or the naturally occurring allergen, proteins having sequence identity greater than 10%, preferably greater than 20% to the allergen can in general be anticipated to have homologous folding patterns and thus be applied as scaffold proteins. However, even protein with amino acid identity less than 10% (exemplified by accession no. AAH05642 and pdb entry 1jss) have been 30 known to have the same folding pattern as the allergen to which is compared e.g. in this case Bet v 1. The three-dimensional structure of the scaffold protein may also be modeled based on the known structure of the allergen in question, or vice versa. More preferably amino acid sequence identity

between the scaffold molecule and the allergen in question should be below 67%, more preferably between 20 and 60%, more preferably between 20 and 50%, and even more preferable 30-50%.

5 A number of amino acids on the molecular surface of the scaffold molecule are identical with amino acids on the surface of the allergen in question, and positioned in corresponding positions in the homologous structure. This can be visualized by comparison of the three-dimensional models or predicted three-dimensional structures.

10 A surface area is selected for "epitope grafting", preferentially an area comprising as many identically positioned amino acid residues in the scaffold and the allergen respectively as possible. The number of artificial mutations needed to fully or partly establish a surface area that mimicks an allergen on 15 the surface of the scaffold protein is thereby minimized. Preferably, the generated surface area comprises between 1000 and 3600 Å², and not larger than 5000 Å². It has been estimated that around 2000 Å² is masked upon binding by the CDR region alone. Furthermore there will be steric hindrance caused by the presence of the entire antibody. Thus, an area of up to 5000 20 Å² is therefore inaccessible to binding by other antibody molecules.

Within the selected area of the scaffold protein, surface amino acids that are different between the scaffold protein and the allergen in question are mutated in such a way that most or preferably all of the surface exposed 25 amino acid residues within the selected area become homologous, or identical to surface exposed amino acids of the allergen in question.

In a further aspect surface areas may be modified to become less identical or 30 homologous to the allergen surface in order to avoid cross-reaction to these surfaces of allergen specific antibodies. In such surface areas, amino acid residues that are identical or homologous to amino acid residues in the corresponding position of the allergen are substituted by residues that are different or less homologous.

The three-dimensional structure of the resulting mutated scaffold protein, i.e. the recombinant protein variant may be verified with respect to its overall folding pattern as well as the conformation of the "grafted" epitope area. Methods of determining secondary and tertiary structures of a protein include

5 X-ray crystallography, NMR spectroscopy, and circular dichroism spectroscopy. The stability of the resulting mutated molecule may be assessed by circular dichroism spectroscopy applying a range of variations in urea concentration, pH, ion strength, temperature, or other parameters.

10 In a vaccination scenario, protein variants or preferentially a mixture of protein variants with non-overlapping allergen specific surface areas, are expected to be safe, since two IgE antibodies specific for the allergen in question preferably cannot bind simultaneously, and hence do not trigger allergic reactions. Furthermore they are expected to be efficient in stimulating

15 protective IgG antibody responses, wherein the IgGs are cross-reactive with the natural allergen in question, within surface areas covered by the allergen specific surface areas.

20 According to the other aspect of the invention, mutated protein variant with several grafted areas distributed over parts of or the entire molecule are generated. Scaffold proteins may be mutated so as to reduce or increase antibody binding affinity or both, thereby eliminating fully or partly epitope areas and/or establishing epitopes areas on other parts of the scaffold protein. The idea is to achieve a general low affinity interaction over the

25 entire surface with respect to binding of allergen specific IgE antibodies.

30 A number of amino acid residues on the surface of the scaffold protein are identical with amino acid residues on the surface of the allergen in question. This can be visualized by comparison of the three-dimensional structures of the allergen and the scaffold protein.

The surface areas that are selected for reducing IgE affinity of the scaffold protein are coherent surface areas comprising amino acid residues that are homologous or identical between the allergen and the scaffold protein. Such

amino acid residues are then mutated to amino acid residues that are related or non-related to amino acid residues in the allergen in question.

5 In surface areas that are selected for increasing the allergen specific antibody cross-reactivity of the protein variant, surface amino acid residues, which are different comparing the scaffold molecule and the allergen are selected. These amino acid residues are then mutated to amino acids that are homologous, or more preferably identical, with the allergen in question.

10 The surface of a recombinant protein variant(-s) comprises areas of amino acids that are identical as well as areas of amino acids that are non-identical with the allergen in question. When the surface of the recombinant protein variant is graphically represented, it can be visualized that the surface comprises at least one essentially circular area that is homologous or 15 identical with the corresponding area of the naturally occurring allergen. This area covers about 300-900 Å², more preferably 500-800 Å², and most preferably 500-600 Å².

20 Accordingly, preferably the scaffold protein does not have any immunogenic cross-reactivity with the naturally occurring allergen. However, it is possible to use a scaffold protein having immunogenic cross-reactivity with the natural occurring allergen, in which case preferable one or more of the epitopes are modified in order to eliminate or decrease the antibody binding affinity of these epitopes.

25 In other words, the main aspect of the present invention relates to a recombinant protein variant with the ability to induce a protective immune response to a naturally occurring allergen, wherein the protein variant

30

- is a variant of a scaffold protein, said scaffold protein has a three-dimensional folding pattern that is structurally similar to that of the naturally occurring allergen,
- compared to the scaffold protein, comprises two or more primary mutations spaced by at least one non-mutated amino acid residue,

each primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to the corresponding amino acid residue or residues in the naturally occurring allergen, and

- has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.

5 A primary mutation according to the present invention might comprise a substitution a deletion and/or an addition. According to the invention, 2-50, preferably 2-40, more preferably 3-25, more preferably 4-15 and most 10 preferably 5-12 primary mutations are performed. It is a very important aspect of the present invention that at least two primary mutations are performed, ensuring that three-dimensional epitope areas are established that can bind allergen specific antibodies are grafted onto the scaffold molecule.

15 The present invention furthermore comprises protein variants with one or more secondary mutations introducing into the scaffold protein amino acid residues, which are not present in the corresponding position in the natural occurring allergen. The purpose of introducing secondary mutations may e.g. 20 be to stabilize the three-dimensional folding pattern of the protein variant or to decrease allergen specific IgE cross-reactivity.

According to the invention, 0-20, preferably 1-10, or most preferably 2-5 secondary mutations are performed.

25 In general, secondary mutations according to the present invention may be performed as a mutation of one or more amino acid residues with any amino acid residue that is not present in the particular position in either the scaffold protein or the naturally occurring allergen. Preferably the secondary mutation 30 is a substitution of an amino acid residue that is not present in the naturally occurring allergen but is homologous to the scaffold amino acid in order to minimize the risk of destabilizing the protein variant.

A primary or secondary mutation according to the present invention may comprise mutations carried out by site-directed mutagenesis (insertion, deletion or substitution), DNA shuffling, and/or by gene library methods.

- 5 Any primary or secondary mutation according to the invention may consist of a number of consecutively mutated amino acids. 1-15, preferably 1-10 and most preferably 1-5 consecutive amino acids are mutated in a primary or secondary mutation according to the present invention. In many cases the surface exposed amino acids that are part of an epitope area are present as 10 single amino acid "points" within the primary structure of an allergen. In such cases it is usually sufficient to point mutate such amino acids. However, in other cases it is necessary to mutate several consecutive amino acids in order to increase or decrease antibody binding affinity.
- 15 An aspect of the invention comprises a method of producing a recombinant protein variant with the ability to induce a protective immune response to a naturally occurring allergen, comprising the steps of:
 - 20 • selecting a scaffold protein, said scaffold protein having a three-dimensional folding pattern that is structurally similar to that of the naturally occurring allergen,
 - introducing two or more primary mutations, that are spaced by at least one non-mutated amino acid residue, into the scaffold protein, each primary mutation introducing into the scaffold protein at least one 25 amino acid residue identical or homologous to the corresponding amino acid residue or residues in the naturally occurring allergen, and
 - thereby producing a protein variant that has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.
- 30 A product obtainable by the above method is also included within the scope of the present invention.

In one embodiment, a protein variant according to the present invention compared with the scaffold protein has an increased binding capacity with respect to antibodies specific to the naturally occurring allergen. Said binding capacity is preferably increased to at least 10 %, preferably to least 50 %,

5 and up to 100 % of the antibody binding capacity of the natural allergen. Said protein variant furthermore preferably has a reduced ability to induce histamine release compared to the naturally occurring allergen. The reduction in histamine release potential of the protein variant is preferably reduced by 2 fold, more preferably by 10 fold, even more preferably by 100 fold, even more preferably by 1000 fold and most preferably by a 10,000 fold or more reduction compared with the ability of the naturally occurring allergen. By ensuring that the ability of the protein variant to induce histamine release is greatly reduced i.a. the ability of the variant to cross-link the IgE receptor the protein variant thus constitutes a more safe alternative to the natural allergen as the active component of an allergen vaccine, since the ability to cause histamine release is largely associated with and responsible for adverse effects associated with the immediate type allergy response and allergy vaccination.

10

15

20 According to a second embodiment, a protein variant according to the present invention has a three-dimensional folding pattern or α -carbon backbone tertiary structure that closely resembles that of the scaffold protein as measured as described in Example 4. The surface contour making up an epitope is supported by the folding pattern of the peptide α -carbon backbone.

25 According to a preferred embodiment a protein variant according to the invention is therefore preferably derived from a scaffold protein that has a level of amino acid identity with the naturally occurring allergen of between 20 and 60 %, preferably between 30 and 50 %.

30 According to a third embodiment, a protein variant according to the present invention has all the primary mutations located in a surface region having an area of about 600-900 A^2 so as to ensure that the primary mutations constitute an epitope area that can be recognized by the complementarity

determining region of an allergen specific antibody. Furthermore, a protein variant according to the invention preferably comprises primary mutations of surface-exposed amino acids. A surface exposed amino acid has a solvent accessibility of above 20 %, preferably above 30 %, more preferably above 5 40 %, and most preferably above 50 %.

According to a fourth embodiment of the present invention, the naturally occurring allergen is an inhalation allergen, preferably originating from the taxonomic order of *Fagales*, *Oleales* or *Pinales*, and most preferably Bet v 1 10 (database accession number: Z80104). A preferred Bet v 1 scaffold protein is Mal d 1.

In other preferred embodiments, scaffold proteins of the major birch pollen allergen Bet v 1 are e.g. isoforms of Aln g 1 (alder) exemplified by accession 15 number S50892, isoforms of Car b 1 (hornbeam) exemplified by Q96382, Q96381, CAA47367, Q96377, Q96378, Q96379, Q96503, Q96501, CAA47366, Q96380, isoforms of Fag s 1 (beech) exemplified by AJ130889, isoforms of Cas s 1 [*Castanea sativa*] exemplified by AJ417550, isoforms of 20 Mal d 1 (apple) exemplified by accession numbers AJ488060, Q43550, Q43551, Q43552, Q43549, AAK13027, AAK13028, Q40280, AAD13683, Q9SYV2, Q9SYV3, Q9SYV4, Q9SYV6, Q9SYV9, Q9SYW3, Q9SYV5, AAK13029, Q9SYV7, Q9SYV8, JC4276, S51119, isoforms of Pru av 1 25 (cherry) exemplified by accession numbers O22521 O24248, O50001 and gi13787043, isoforms of Pyr c 1 (pear) exemplified by accession numbers AF057030, isoforms of Api g 1 (celery) exemplified by accession numbers p49372, p92918, isoforms of Dau c 1 (carrot) exemplified by accession 30 numbers O04298, t14322, t14325, isoforms of Lupin exemplified by accession number p52779 (pdb entry 1IFV_A), isoforms of other Patogenesis Related proteins exemplified by accession numbers CAA10719, S20517, P93333, AB17_PEA, AB18_PEA, T14817 and other proteins that contains the same arrangement of secondary structure elements exemplified by accession number AAH05642 (pdb entry 1jss).

In an embodiment, where Mal d 1 (2620) (database accession number: AJ488060) is the Bet v 1 scaffold protein, a protein variant comprises at least two primary mutations selected from the group consisting of: (E12V, E12I, E12M, E12L), P16A, (H40S, H40T), I43N, L44I, D47N, G65K, K70R, (E76H, E76R, E76K, Q76H), S107T, G108P, +109D, S110G, E129A, K152L, (P154S, P154T), P155S and optionally one or more secondary mutations are selected from the group consisting of: N28X, preferably N28T, K32X, preferably K32Q, E45S, E96X, +159X. In this context, as well as in the following examples, the first letter and number corresponds to the original amino acid code in a particular amino acid position. The following letter is the amino acid that can be substituted with the original amino acid. Parenthesis means that there are a number of different options when choosing to mutate in a given amino acid position. One of these can be chosen.

15 In a specific embodiment, a Mal d 1 protein variant (rMal d 1 (2760)) comprises the sequence defined in SEQ ID NO 1:

16 G V Y T Y E N E Y T S E I P P P R L F K A F V L D A D T L I P Q I A P Q A I K H A E I L S G D G G P G T I K
17 K I T F G E G S Q Y G Y V K H K I D S V D E A N Y S Y A Y T L I E G D A L T D T I E K V S Y E T K L V A S
18 G S G S I I K S I S H Y H T K G D V E I M E E H V K A G K E K A H G L F K L I E S Y L K D H P D A Y N,

19 said variant comprising the following secondary mutations: N28T, K32Q, E45S. The rMal d 1 (2760) variant is an example of a scaffold protein according to the present invention that comprises a number of secondary mutations that together decrease the Bet v 1 specific IgE reactivity of the scaffold protein, as illustrated in detail in the examples.

20 In another specific embodiment, a protein variant of a Mal d 1 protein variant (rMal d 1 (2781) comprises the sequence defined in SEQ ID NO 2:

21 G V Y T Y E N E Y T S E I P P P R L F K A F V L D A D N L I P Q I A P Q A I K H A E N I E G N G G P G T I K
22 K I T F G E G S Q Y K Y V K H R I D S V D H A N Y S Y A Y T L I E G D A L T D T I E K V S Y E T K L V A S
23 G S G S I I K S I S H Y H T K G D V E I M E E H V K A G K E K A H G L F K L I E S Y L K D H P D A Y N,

said variant comprising the following primary mutations: I43N, L44I, D47N, G65K, K70R, Q76H. The rMal d 1 (2781) variant is an example of a protein variant according to the present invention that has increased Bet v 1 specific IgE reactivity in comparison with the "native" Mal d 1 2620. This is illustrated 5 in detail in the Examples.

In yet another specific embodiment, a protein variant of a Mal d 1 protein variant (rMal d 1 (2762)) comprises the sequence as defined in SEQ ID NO 3:

10 GVYTYENEYTSVIPPARLFKAFVLDADNLIPKIAPIQAIKHAEILEGDGGPGTIK
KITFGEGSQYGYVKHKIDSVDEANYSYAYTLIEDALTDIEKVSYETKLVAT
PDGGSIIKSISHYHTKGDVEMEEHVKAGKEKAHGLFKLIESYLLDHSDAYN,

15 said variant comprising the following mutations: E12V, P16A, K152L, P155S, S107T, G108P, +109D, S110G. "+" means in this connection insertion of an amino acid at the indicated position. The rMal d 1 (2762) variant is an example of a protein variant according to the present invention that has increased Bet v 1 specific IgE reactivity in comparison with the "native" Mal d 1 2620. This is illustrated in detail in the Examples.

20 In an embodiment demonstrating that a protein variant of Mal d 1 may comprise both primary and secondary mutations, such a protein variant comprises at least two primary mutations selected from the group consisting of: (E12V, E12I, E12M, E12L), (H40S, H40T), (E76H, E76R, E76K), E129A, (P154S, P154T), and optionally one or more secondary mutations selected 25 from the group consisting of: E8X, N28X, K32X, E96X, +159X.

According to a fifth embodiment a protein variant of the present invention is a 30 protein variant wherein Dau c 1 (database accession number: T14325) is a scaffold protein of Bet v 1. According to this embodiment, a protein variant of Dau c 1 comprises at least two primary mutations selected from the group consisting of: (S12V, S12L, S12I, S12M), S14P, E16A, P105A, A107P, (A148S, A148T), (I151L, I151V, I151M), (N153H, N153K, N153R), (+154S,

+154T), (+155D, +155E), +156A, (+157Y, +157F), (+158N, +158Q), (K39S, K39T), (K44E, K44D), (V52I, V52M, V52L), (I54K, I54R, I54H), (T64K, T64R, T64H), (T65Y, T65F, T65W), (T67K, T67R, T67H), D86E, L91G, (G92D, G92E) and optionally one or more secondary mutations are selected from the 5 group consisting of: K32X, E42X, E59X, R69X, E95X, K122X, E8X, T10X, D25X, K32X, D46X, E59X, E95X, D108X, K122X.

In a specific embodiment, a Dau c 1 protein variant comprises at least two primary mutations selected from the group consisting of: (S12V, S12L, S12I, 10 S12M), S14P, E16A, P105A, A107P, (A148S, A148T), (I151L, I151V, I151M), (N153H, N153K, N153R), (+154S, +154T), (+155D, +155E), +156A, (+157Y, +157F), (+158N, +158Q) and optionally one or more secondary mutations selected from the groups consisting of: K32X, E42X, E59X, R69X, E95X, K122X. This embodiment is a demonstration of establishment of a 15 single epitope area on the surface of a scaffold protein while other areas have been mutated so as to decrease antibody affinity.

In another specific embodiment, a Dau c 1 variant comprises comprises at least two primary mutations selected from the group consisting of: (K39S, 20 K39T), (K44E, K44D), (V52I, V52M, V52L), (I54K, I54R, I54H), (T64K, T64R, T64H), (T65Y, T65F, T65W), (T67K, T67R, T67H), D86E, L91G, (G92D, G92E) and optionally at least one secondary mutation is selected from the group consisting of: E8X, T10X, D25X, K32X, D46X, E59X, E95X, D108X, K122X. This embodiment is another example of establishment of a single 25 epitope area on the surface of a scaffold protein and decrease of antibody affinity of other parts of the molecule.

In a sixth embodiment of the present invention, the naturally occurring allergen originates from the taxonomic order of *Poales*, *Asterales* or 30 *Urticales*. Examples of scaffold proteins of the major grass pollen allergen *Phl p 1* are isoforms of *zea m 1* exemplified by accession number Q07154, isoforms of *Gly m 1* exemplified by accession number U03860, isoforms of *Ory s 1* exemplified by accession number U31771, isoforms of β -expansins exemplified by accession numbers: u95968, AC001229, U95967, s53082,

U30477, U85246, Y07782, U30479, U30481, U30480, U30478, U30476, U30460, U30382, U64890, U64891, U64892, U64893, U82123, D26459, D88415, isoforms of Group 2 and group 3 grass allergens exemplified by accession numbers: P14947, X73363, A48595, P43214, P14948, U25343, 5 Z50867. *Phl p 5* is another example of a grass pollen allergen. Examples of *Phleum pratense* group 5 allergen, *Phl p 5* scaffold proteins include: P93466, O81342, Q9SBE0, O81343, O81344, 2023228A, S38584. Examples of *Lolium perenne* group 5 allergen, *Lol p 5* scaffold proteins include CAB64344, Q9XF24, a38582. Examples of *Poa pratensis* group 5 allergen, 10 *Poa p 5* scaffold proteins include: Q9FPR0, B39098. Isoforms of *Holcus lanatus* group 5 allergen, *Hol l 5* exemplified by (O23971, O23972, Q9FPQ9). Isoforms of *Phalaris aquatica* group 5 allergen, *Pha a 5* exemplified by (MP51_PHAAQ). Isoforms of *Dactylis glomerata* group 5 allergen, *Dac g 5* exemplified by (Q93XE0, Q93XD9). Isoforms of *Hordeum vulgare* group 5 15 allergen, *Hor v 5* also referred to as *Hor v 9* exemplified by (O04828). Isoforms of *Phleum pratense* group 6 allergen, *Phl p 6* exemplified by (O65868, CAA76557).

20 In a seventh embodiment of the present invention, the naturally occurring allergen is a dust mite allergen, preferably originating from *Dermatophagoides*, and preferably *Der p 2* (database accession number: P49278). Preferred *Der p 2* scaffold proteins are *Eur m 1* (database accession number: P25780), *Gly d 2* (database accession number: AJ272216), and *Lep d 2* (database accession number: S66499).

25 A specific embodiment of a *Lep d 2* protein variant is a protein variant that comprises at least two primary mutations selected from the group consisting of: D17L, D17I, D17V, D17M, S19P, Q32K, Q32R, Q32H, K33P, T35Q, T35N, N88K, N88R, N88H, T92N, T92Q, A95K, A95R, A95H and optionally 30 one or more secondary mutations selected from the group consisting of: K6X, S22X, R30X, K76S, K81X, V114X. This is an example of a *Lep d 2* protein variant with a single epitope area established on the surface of the protein,

optionally with downregulation of Der p 2 antibody cross-reactivity at other parts of the surface.

Another specific embodiment of a Lep d 2 protein variant is a protein variant
5 that comprises at least two primary mutations within a single epitope area
selected from the group consisting of: D45N, D45Q, N47K, N47R, N47H,
K48T, K48S, T50K, T50R, T50H, K52E, K52D, L54K, L54R, L54H, E107K,
E107R, E107M, H112D, H112E, T119I, T119L, T119V, T199M and optionally
10 one or more secondary mutations selected from the group consisting of: K6X,
S22X, K29X, R30X, K76X, K81X.

A specific embodiment of a Gly d 2 protein variant is a protein variant that
comprises at least two primary mutations selected from the group consisting
of: K2Q, K2N, K4D, K4E, K10N, K10Q, T14K, T14R, S22H, S22R, S22K,
15 K39V, K39L, K39I, K39M, D45N, D45Q, T60L, T60V, T60I, T60M, Q6three-
dimensional, Q63E, K80V, K80L, K80I, K80M, T91S, H112D, H112E, R122I,
R122V, R122L, R122M and optionally one or more secondary mutations
selected from the group consisting of: K6X, preferably K6R or K6H, R30X,
preferably R30K or R30H, F74X, preferably F74Y or, F74W, K81X, preferably
20 K81R or K81H, K88X, preferably K88R or K88H, T90X, and V114X,
preferably V114L, V114I or V114M. This is an example of a protein variant
with increased IgE reactivity in comparison with Der p 2 and optionally with
secondary mutations where amino acids are substituted with homologous
amino acids in order to avoid destabilization of the protein variant.

25 Other specific embodiments of Der p 2 scaffold proteins are isoforms of
group 2 dust mite *Tyrophagus putrescentiae* allergens Tyr p 2 exemplified by
accession number O02380, isoforms of storage mite *Lepidoglyphus*
destructor group 2 allergens Led d 2 (mistakenly referred to as Led d 1 in
30 some publications) exemplified by accession numbers: P80384, S48727,
2118249B, isoforms of Gly d 2 exemplified by accession numbers:
CAB76459, Q9U5P7, isoforms from the group of homologous proteins
exemplified by accession numbers O97763, P79345, Q9VQ62, Q9Z0J0,
AAF99719, Q28895.

In a further preferred embodiment scaffold proteins of the major house dust mite allergen Der p 1 is Der f 1 (accession numbers P16311).

5 In an eighth embodiment of the present invention the naturally occurring allergen is a insect allergen from cockroach (*Blatella germanica* (allergens: Bla g 1 or Bla g 2), *Periplaneta Americana* (allergen: Per a 1) or midges (*Chironimus Spp.* – allergen: Chi t 1)

10 In a ninth embodiment of the specific invention, the naturally occurring allergen is an animal allergen, preferably a mammalian allergen, preferably originating from a cat, dog, rat, mouse or horse, preferably the Fel d 1 allergen from cat.

15 In a tenth embodiment of the present invention, the naturally occurring allergen is a venom allergen, preferably originating from the taxonomic order of *Hymenoptera*, *Vespidae*, *Apidae*, or *Formicoidae*. A preferred venom allergen is *Ves v 5*. Possible *Ves v 5* scaffold proteins include: P35783, P35760, P35785, P35784, P35787, P35736, P35786, P10737, Q05108, P35781, P35782, P81657, P81656, P35780, P35759, Q05109, P35779, 20 P35778). Furthermore other low homologous proteins exemplified by (P54108, O19010, P16562, Q16937, P48060, Q60477, P35795, P35795, Q09566, P47033, Q40374, P12020, Q41359, P47032, Q91055, Q034401, P08299, P11670, P36110, P16563, P54107, Q08697, Q04108, P79845, P07053, P09042, P35794, P04284, Q03402, P33154, P35792, Q05968, 25 P35793, Q00008, Q41495, P16547, P13390, O22456, Q15335, Q62871, O88487, Q13409, P54131, Q9LRZ5, P18859, O04067.

30 In an eleventh embodiment of the present invention, the naturally occurring allergen is a food allergen from peanut, soya, cows milk, hens egg white or yolk, shrimp or cod.

In a twelfth embodiment of the present invention, the scaffold protein is homologous to a plant, grass, food, or mite allergens. Protein variants according to this embodiment comprise at least one primary mutation.

Protein variant furthermore have deconvoluted CD-spectra that deviate less than 30%, preferably less than 20%, and even more preferably less than 10% compared to the deconvoluted CD-spectra of the naturally occurring allergen. Preferably, the scaffold protein has a level of sequence identity with 5 the naturally occurring allergen of between 30 and 50 %, and preferably, the α -carbon backbone tertiary structures of the scaffold protein and the natural occurring allergen resemble each other to the extent that the deviation between their circular dichroism spectra should deviate by less than 30%, preferably less than 20%, and even more preferably less than 10 (Example 10 4).

In thirteen's preferred embodiment of the present invention, the naturally occurring allergen is fungi protein.

15 Preferably, a protein variant of the invention comprises at least one T-cell epitope capable of stimulating a T-cell clone or T-cell line specific for the naturally occurring allergen.

Accordingly, a protein variant preferably induces a new allergen specific 20 Th0/1 T-cell immune response on top of the Th2 T-cell response or the scaffold protein variants induce T-cells anergy or a shift of the response of the T-cells from the Th2-type to the Th1-type

25 The invention further relates to use of protein variants according to the present invention for use as a pharmaceutical as well the manufacture of a medicament for the treatment or prevention of allergy. Preferably, a pharmaceutical composition comprises two or more different recombinant protein variants according to the present invention, wherein each variant is defined by having at least one primary mutation, which is absent in at least 30 one of the other variants. In this way, presentation of different epitope areas that mimick epitopes of the naturally allergen can be achieved while ensuring that the risk of IgE cross-binding on the surface of e.g. mast-cells is minimized. A composition according to the present invention preferably comprises 2-12, preferably 3-10, more preferably 4-8, and most preferably 5-

7 different protein variants. Such compositions can be used for preparing a pharmaceutical for preventing and/or treating allergy.

Pharmaceutical compositions according to the present invention preferably further comprise a physiological or a pharmaceutically acceptable carrier and/or excipient and/or an adjuvant. "Carriers or excipients include any carrier or excipient commonly used with pharmaceuticals such as oils, starch, sucrose and lactose. The compositions according to the present invention may be in a form suited for oral intake including, capsules, pills, tablets and syrups. Alternatively, compositions according to the present invention may be in the form of a parenteral formulation suitable for e.g. intravenous, subcutaneous, etc. administration. "Adjuvants" refers to a substance that stimulates and prolongs antibody synthesis when injected together with an antigen. An example of an adjuvant includes Freunds complete/incomplete adjuvants and aluminium hydroxide gels. A pharmaceutical composition according to the present invention is preferably in the form of a vaccine against allergic reactions elicited by a naturally occurring allergen in patients suffering from allergy.

Included in the scope of the present invention is methods of vaccination or treatment of an individual by generating an immune response in the individual comprising administering to the subject a recombinant protein variant or a pharmaceutical composition according to the invention.

Also comprised in the scope of the present invention are processes for preparing a pharmaceutical composition mixing a recombinant protein variant or a composition according to the present invention with pharmaceutically acceptable substances and/or excipients and/or adjuvants.

A further aspect of the present invention includes methods of preparing a recombinant protein variant wherein the recombinant protein variant is produced from a DNA sequence obtained by DNA shuffling (molecular

breeding) of DNA sequences encoding the scaffold protein and the naturally occurring allergen.

Finally the present invention relates to DNA sequences encoding recombinant protein variants. Likewise, the present invention comprises expression vectors and host-cells that contain DNA sequences according to the present invention and are capable of producing recombinant protein variants. Also, methods of producing recombinant protein variants comprising the step of cultivating such host-cells with vectors are included in the present invention. Diagnostic assays for assessing relevance, safety, or outcome of therapy of a subject using a recombinant protein variant are also included in the invention, wherein an IgE containing sample of the subject is mixed with said protein variant or said composition and assessed for the level of reactivity between the IgE in said sample and said protein variant.

15

DEFINITIONS

Scaffold protein: The scaffold protein is a naturally occurring protein that often has a significant degree of amino acid sequence homology with the allergen that is selected for allergy vaccination. Furthermore, the scaffold protein has a three-dimensional folding structure resembling that of the naturally occurring allergen. It is difficult to give an exact definition on which criteria must be fulfilled in order for two proteins to have a similar folding pattern. However, the man skilled in the art knows that proteins or protein domains with more than 10% amino acid homology have a high probability of being folded in a similar way and sharing similar amounts of α -helices, random coil, and β -sheets. This can be measured by e.g. circular dichroism, wherein the deviation between the two protein spectra should deviate by less than 30%, preferably less than 20%, and even more preferably less than 10.

30

Preferably, there is little or no measurable IgE cross-reactivity between the naturally occurring allergen and the scaffold protein, thereby enabling establishment of an allergen specific surface area, i.e. an epitope structure on the scaffold protein. A mutated scaffold protein with only one allergen

specific epitope area is thus unable to bind more than one IgE molecule and thus unable to cause cross-binding of IgE molecules attached to the surface of e.g. mast-cells. Protein variant surface areas that resemble allergen epitopes are herein referred to as e.g. "epitope area", "mimicking an epitope", 5 etc.

The degree of sequence homology between the scaffold protein and the allergen is between 0 and 95%, preferably between 5 and 90%, more preferably between 10 and 67%, even more preferably between 20 and 60 10 %, and most preferably between 30 and 50 %.

67% is often chosen as the lowest degree of sequence identity in order to characterize two proteins as isoforms or homologous proteins (King et al, 1995) J. Allergy Clin. Immunol. 96:5-14). The scaffold protein is preferably 15 not an isoform or a homologous protein of the naturally occurring allergen, thereby ensuring that the scaffold protein has a low degree of antibody cross-reactivity with the allergen.

The protein variant compared to the scaffold protein has an increased 20 binding capacity with respect to antibodies that are specific to the naturally occurring allergen: This statement can be regarded as both relating to the complete protein variant as well as to a single epitope.

Scaffold protein devoid of cross-reactivity with the naturally occurring 25 allergen: The scaffold molecule selected for mutation may be devoid in binding antibodies specific for the allergen in question, or preferably some IgE antibodies from an average patient allergic to the allergen in question may be cross-reactive. Devoid in antibody binding means a reduction in antibody affinity of a factor of 10^3 , more preferably 10^4 , most preferably 10^5 or 30 more.

Primary mutations (creation of epitopes, stabilizing backbone, etc.)
"Introduction of a primary mutation" may be a substitution, a deletion or an addition. Preferably the primary mutation is a substitution of at least one

scaffold amino acid residue with residue (-s) identical or homologous to the amino acid found in a corresponding position in the natural allergen. Preferably, the protein variant comprises 2-50, more preferably 2-40, more preferably 3-25, more preferably 4-15 and most preferably 5-12 primary mutations. Also, the protein variant may comprise one or more mutated sequence fragments consisting of a number of consecutive mutated amino acids. Preferably, the mutated sequence fragment consists of 2-15, more preferably 3-12 and most preferably 5-10 amino acids.

10 In a preferred embodiment of the invention, all primary mutations of the protein variant are located in a surface region having an area of 900 Å². In this preferred embodiment of the invention the protein variant only has one epitope with cross-reactivity with the naturally occurring allergen. It is believed that such a protein variant has the advantage that it will not give rise to an undesirable immune response, e.g. an anaphylactic reaction, since such responses require IgE cross-linking to be triggered, i.e. such responses require that the allergen has at least two non-overlapping epitopes.

15 Preferably, the primary amino acid residues to be mutated are surface-exposed amino acids. It is preferred that the amino acid residues to be mutated have a solvent accessibility of above 20 %, preferably above 30 %, more preferably above 40 %, and most preferably above 50 %.

20 In addition to or as an alternative to mutation of surface exposed amino acids, i.e. the amino acid constituting the epitopes, the amino acids of the backbone of the protein, i.e. amino acids located in the interior of the tertiary protein structure may be mutated. Such mutations can potentially modify the folding of the molecule and thereby change the binding properties of the epitopes on the surface of the protein. One purpose of the mutations may be 25 to stabilise the folding of the backbone and hence the tertiary structure of the protein, e.g. by introducing sulphur bridges.

30 In one embodiment of the invention, one or more of the mutations are carried out by site-directed mutagenesis.

In a second embodiment of the invention, one or more of the mutations are carried out by DNA shuffling.

5 In a third embodiment of the invention, one or more mutations are introduced by a semi-random library methodology.

Secondary mutations (weakening/elimination of epitopes): The secondary amino acid mutations are mutations introducing into the scaffold protein 10 amino acid residues, which are not present in the corresponding position in the naturally occurring allergen.

The secondary amino acid mutation may be a substitution, a deletion or an addition. Preferably, the protein variant comprises 0-50, more preferably 1-15 40, more preferably 2-25, more preferably 3-15 and most preferably 4-12 secondary mutations. Also, the protein variant may comprise one or more secondary mutations each consisting of a number of consecutive mutated amino acids. Preferably, the mutated sequence fragment consists of 2-15, more preferably 3-12 and most preferably 5-10 amino acids.

20 The purpose of the secondary mutation as mentioned above in connection with the scaffold protein is to modify an epitope of the scaffold protein to eliminate or reduce its antibody binding effect, and hence IgE cross-linking and risk of inducing anaphylactic reactions.

25 Epitope grafting: Epitope grafting is understood as establishment of allergen specific antibody binding properties on the surface of a scaffold protein. This "grafted" scaffold protein variant represents an attractive vaccine candidate against allergies due to the capacity of raising a protective immune response.

30 Protective immune response: Raising a protective immune response means to alter the reaction of the immune system towards a naturally occurring allergen in order to avoid the adverse effects associated with allergy. The protective immune response is thought to be mediated largely by generation

of a large number of IgG antibodies that presumably block the interaction between allergen and IgE antibodies. A protective immune response most likely also involves stimulation of T-cells.

5 Surface structure/contour: Whereas the three-dimensional folding of a protein is determined by the tertiary folding of the α -carbon backbone, the nature of the molecular surface structures/contours with regard to topography and charge distribution of a protein is in part determined by the nature and composition of amino acid chains that is present at the surface and hence 10 show some degree of solvent accessibility. Introduction of primary and/or secondary mutations at the protein surface will therefore alter the nature of the protein surface structures/contours.

15 Composition of the invention: A composition or a pharmaceutical composition according to the invention may comprise at least one scaffold protein variant as well as any other pharmaceutical or non-pharmaceutical reagents, excipients, adjuvants, carriers, vehicles or drug delivery systems.

20 Protein variant: A protein having an amino acid sequence, which differs from a natural occurring protein by one or more amino acids. Preferably, a protein variant according to the present invention is a recombinant protein variant of a scaffold protein, said protein variant can cross-react with patient sera from individuals that are allergic to an allergen that has significant amino acid sequence homology with a naturally occurring allergen. However, the protein 25 variant has a lower degree of IgE reactivity compared with naturally occurring allergen.

30 Level of amino acid identity: The level of amino acid identity is here defined by the percentage of amino acid residues that in two primary protein sequences with respect to corresponding positions are identical.

Level of amino acid homology: The level of amino acid homologies as herein defined by the percentage of amino acid residues that in two primary protein

sequences with respect to corresponding positions are either identical or homologous amino acid residues.

Allergen: An allergen according to the present invention is any naturally occurring protein that has been reported to induce allergic, i.e. IgE mediated reactions upon their repeated exposure to an individual. Examples of naturally occurring allergens include inhalation allergens (tree- and grass pollen allergens, mite allergens, insect allergens (inhalant and venom allergens), animal hair and dandruff allergens from e.g. dog, cat, horse, etc.), and food allergens.

Modulation of an immune response: Following vaccination with a recombinant protein variant that partly mimicks the structure of the allergen, an "immune response" is thought to occur at several levels: (a) the protein variant may bind to preformed IgE that are either prebound to e.g. masT-cells or found in the circulation, without causing IgE cross-binding on the surface of the masT-cells, (b) novel IgG antibodies may be formed or existing IgG antibodies might become "matured" in order to increase epitope affinity, and (c) novel or existing T-cells may become activated. Modulation of the immune response in the context of the present invention is thus interpreted as the ability of a recombinant protein variant to induce a protective IgG response and preferably also stimulating T-cells while at the same time reducing the risk of IgE cross-binding on the surface of e.g. masT-cells. It is primarily the IgE cross-binding on the surface of masT-cells that is thought to be responsible for the adverse effects following allergen exposure.

It can be measured in several ways whether an allergen variant raises "an immune response corresponding to that of a naturally occurring allergen". Not limiting to the invention a preferred way of measuring this is by measuring in a statistically significant manner ($p < 0.05$) in at least one immunoassay the ability of the scaffold protein variant to bind to serum IgE from an allergic subject or to inhibit the binding of serum IgE from an allergic subject to the natural occurring allergen. Another preferred way is the measurement of the ability of antibodies in a serum sample from scaffold

protein variant immunised animals to compete for the allergen in an immunoassay.

Affinity of antibody-antigen interaction: Specificity and affinity of antibody-antigen interactions are two sides of the same coin. The reason for this is that the binding strength, i.e. the affinity, is determined not only by the attractive forces (enthalpic contribution), i.e. ionic interactions, hydrogen bonds and Van der Waals interactions, but also by the almost complete displacement of water molecules from the interface between the two molecules in the complex (entropy contribution). In common language this means that a perfect fit in the topographical contours of the molecular surfaces is of crucial importance.

Reduced fit, e.g. as a consequence of mutation, will almost always lead to reduced affinity of the interaction. The complex will still be formed, but at lower concentration, and in kinetic terms the equilibrium will be displaced, so that at any given time a higher proportion of the antigen and antibody molecules will be in free solution and conversely a lower proportion of the molecules will be bound in complexes.

Thus, increasing the concentration of any of the reactants, i.e. antigen or antibody, will lead to increased concentration of the complex – regardless of the affinity. In common language this means that any antigen will bind to any antibody provided that the concentrations are high enough. This in turn means that some restrictions should be imposed on the concentrations of the reactants in order to assure that the reaction will take place in concentrations that are close to physiological concentrations, i.e. that we are studying a specific interaction.

It also follows that K_d is a direct measure of the affinity of the interaction. Practically speaking, however, K_d is a difficult measure, since polyclonal antibodies usually will be employed.

If $[AgAb]$ is plotted as a function of $\log [Ag]$ at constant $[Ab]$ an S-shaped curve is obtained. The $[Ag]$ at half maximal binding is a practically useful measure – but of a combination of K_d and B_{max} . The $[AgAb]$ at saturation is a constant, B_{max} , at a given $[Ab]$. B_{max} is conceptually easier to understand since it is the maximal level of binding, however, in practice it can be difficult to achieve high enough concentrations of Ag . B_{max} is the binding capacity. Thus, relative measures of binding capacities at given $[Ab]$ can be compared.

5

An antigen which has been mutated in such a way that a section of the surface is significantly altered has a lower binding capacity with respect to polyclonal antibodies specific for the non-mutated antigen. The average affinity of the interaction between the mutated antigen and a polyclonal antibody is also likely to be reduced. However, if the specificities of the polyclonal antibodies are not randomly dispersed over the entire surface 10 (which is a known situation for some IgE from individual patients), or in the case of a monoclonal antibody, the binding capacity as well as the average affinity may be unaffected by the mutations, i.e. if the epitopes recognized by the antibodies are outside the area affected by the mutations. The chance of this situation is reduced in the case of pools of patient sera or polyclonal 15 antibodies raised in experimental animals by massive immunization.

20

According to the current invention, mutations might be introduced in some surface areas in order to reduce the average affinity in these areas, and vice versa in other, which are different for the purpose of achieving an equalized 25 low-affinity interaction on the entire surface for antibodies specific for the allergen in question. These scaffold protein variant have antibody binding capacity similar to the allergen in question, but with considerably reduced average affinity. The ability to release histamine by cross-linking receptor-bound IgE on the surface of mast-cells or basophils in an in vitro test system 30 can be used to assess these combined features of the scaffold protein variants. The potential to release histamine to some degree reflects the average affinity, and to some degree it mirrors the relevant clinical reaction. Furthermore, such scaffold protein variants stimulate production of IgG antibodies, which will most likely interact with low affinity with the natural

allergen. Due to the great abundance of IgG antibodies in comparison to IgE, even low affinity IgG interactions are thought to have the capacity to interfere beneficially with the high affinity interaction between the natural allergen and a low concentration of IgE.

5

Assesment of IgE reactivity e.g. binding capacity and binding affinity

Experimental approach addressing the evaluation of scaffold protein variant IgE reactivity can be performed in many ways. For instance; K_d , i.e. the affinity of the antibody-antigen interaction, and B_{max} , i.e. the binding capacity, 10 can be experimentally addressed in solid phase immuno-inhibition assays or in direct binding assays.

In solid phase immuno-inhibition assays the antibody may be coupled to the solid phase. The experiments can also be performed using solid phase 15 coupled antigen. The binding of a purified and labeled allergen is then inhibited by a dilution series of the test antigen, i.e. mutated scaffold, and compared to a similar experiment performed using the non-mutated molecule. In a plot of inhibition percentage as a function of the logarithm of inhibitor concentration the relationship corresponds to an S-shaped curve. If 20 a mutated antigen has modified K_d but identical B_{max} this will correspond to a parallel displacement of the curve along the x-axis. A higher affinity will shift the curve to the left and vice verca. A change in B_{max} will affect the maximal level of the curve as well as the slope of the linear section of the S-shaped 25 curve. In all cases where a mutated antigen is compared to a non-mutated molecule, the linear sections of the curves can be statistically tested for linearity and it can be tested whether the linear sections of the curves are parallel, or the comparison between the parameters obtained from a complete curve fit, i.e. four parameter logistic curve fit, can be used for the 30 statistical analysis. If the curves are parallel and displaced relative to each other this is an indication of a different affinity of the antibody-antigen interaction. If the slopes are different this is an indication of a change in epitope composition, i.e. binding capacity or a change in both binding capacity and affinity.

Surface-exposed amino acid: Means that the amino acid residue is located at the surface of the three-dimensional structure in such a manner that when the allergen is in solution at least a part of at least one atom of the amino acid residue is accessible for contact with the surrounding solvent.

5 Preferably, the amino acid residue in the three-dimensional structure has a solvent (water) accessibility of at least 20%, suitably at least 30 %, more suitably at least 40 % and most preferably at least 50 %.

Solvent accessibility: Defined as the area of the molecule accessible to a sphere with a radius comparable to a solvent (water, $r = 1.4 \text{ \AA}$) molecule. The expressions "surface-exposed", "surface amino acid", and "solvent-exposed" are used interchangeably.

10 Homologous amino acid residues: Homologous amino acid residues are characterised by sharing essentially the same chemical features. An example of grouping amino acids is to characterize Ala, Val, Leu, Ile, and sometimes Gly and Pro as "aliphatic residues". Other "groups" include hydroxyl residues (Ser and Thr), acidic residues (Asp and Glu), amide residues (Asn and Gln), basic residues (Lys and Arg), and the aromatic residues (Phe, Tyr, and Trp).

15 20 Other groupings of amino acids into groups sharing similar chemical characteristics are of course possible, e.g. division of amino acids into hydrophobic and hydrophilic residues.

25 DNA shuffling: DNA shuffling is a term that encompasses a number of recombinant methods whereby a number of sequences or sequence fragments that share significant homologies are disrupted by e.g. digestion, sonication, etc. By religation and subsequent PCR (polymerase chain reaction) a large number of novel variants are generated on a random basis. DNA shuffling can also be carried out in a more controlled manner, by e.g. 30 introducing primers with well defined sequences into the reaction.

Gene library methods: Gene library methods include construction, expression and screening of any form of DNA library encoding mutated variants of a protein. The protein coding DNA sequence can either be mutated semi-

randomly or randomly. A non-limiting example that describes a semi-random library could be a library where mutated protein variants are mutated with respect to surface exposed amino acid residues only. Another example is any restraints in respect to the outcome of the mutation i.e. if mutation only 5 allows for substitution to certain amino acid residues i.e. homologous amino acid residues, small or charged/non-charged amino acid residues. In one example the diversity is introduced by the use of degenerated oligo nucleotide primers and PCR- techniques. In another example the mutation diversity is introduced by use of a host organism that introduces mutations at 10 a high frequency.

The present invention is further illustrated by the following non-limiting examples.

15 EXAMPLES

EXAMPLE 1

Modification of scaffold proteins homologous to Bet v 1.

20 Site directed mutagenesis.
All Mal d 1 mutants were generated as follows. First (I), each single mutation (or several mutations if located closely together in the DNA sequence) were introduced into DNA sequences coding for Mal d 1 (2620) (accession number: AJ488060) using sense and anti-sense mutation-specific oligonucleotide primers accommodating each mutation(s) along with sense and anti-sense oligonucleotide primers accommodating either upstream or downstream neighbour mutations or the N-terminus/C-terminus of Mal d 1, respectively as schematically illustrated in Figure 1 (I). Secondly, PCR 25 products from PCR reactions (I) were purified by agarose gel electrophoresis and PCR gel purification, mixed and used as templates for an additional PCR reaction (II) with oligonucleotide primers accommodating the N-terminus and C-terminus of Mal d 1 as schematically illustrated in Figure 1 (II). The PCR 30 products were purified by gel electrophoresis and PCR gel purification

followed by ethanol precipitation, cut with restriction enzymes (Sac / EcoRI) or (Sacl / XbaI), and ligated directionally into pMAL-c restricted with the same enzymes.

5 Nucleotide sequencing.

Plasmid DNA from 10 ml of bacterial culture grown to saturation overnight in LB medium supplemented with 0.1 g/l ampicillin were purified on tip 20 columns (QIAGEN) and sequenced using the Ready reaction dye terminator cycle sequencing kit and a Fluorescence Sequencer AB PRISM 377, both 10 from (Perkin Elmer), following the recommendations of the suppliers.

Protein expression and purification.

Recombinant Mal d 1 (2620) and mutants were over-expressed in Escherichia coli DH 5 α cells in operon with the amino-terminal E. coli fusion 15 partner, maltose binding protein (MBP). Six 5L flasks containing 1L of LB- medium supplemented with 100mg/L ampicillin were inoculated with 10 ml of overnight cultures of transformed E. coli cells grown with shaking at 37°C to an optical density of 0.75 at 600 nm before induced by addition of 5 ml 100mM of IPTG and harvested by centrifugation 3 hours post induction. Cells 20 from 6L of culture were re-suspended in 2x 150 ml lysis buffer (30 mM NaCl, 10 mM EDTA, 1mM Benzamidine, 10 μ g/ml SBTI 10 mM Na2HPO4·12 H2O/NaH2PO4·H2O buffer pH 7.0, 0.02 % NaN3) added 3mM PMSF and lysed by sonication for 10 minutes on ice. After sonication removal of DNA 25 was performed by adding 1.5 ml 1mg/ml DNase, 5mM MgCl2 and 40 ml 4M NaCl before 30 minutes incubation at room temperature. Cell lysates were centrifuged 30 minutes 12000 rpm, 4 C. Supernatants (300 ml) were pooled and filtered using 0.45 μ m filters before addition 1200 ml of dilution buffer (0.5 M NaCl, 2 mM EDTA, 1 mM Benzamidine, 10 μ g/ml SBTI, 10 mM Na2HPO4·12 H2O/NaH2PO4·H2O buffer pH 7.0, 0.02 % NaN3). 30 Recombinant fusion protein was isolated by amylose affinity chromatography by operating 200 ml amylose columns with the Äkta explorer system (Pharmacia Biotech, Sweden) equipped with the Unicore 4.0 software. Eluted fusion protein was concentrated using amicon pressure cells equipped with

30 kDa cutoff filters to 30 ml and dialyzed against 2x 5 L 20 mM TRIS-HCl pH 8.0 overnight.

All MBP-Mal d 1 fusion proteins, were cleaved at 20 °C for 20 hours with slow stirring with 1 % 1mg/ml Factor Xa in cleavage buffer (20 mM TRIS-HCl pH 8.0, 100 mM NaCl, 2 mM CaCl₂, 0.02% SDS) to 1.00% at fusion protein concentrations between OD₂₈₀ = 4-6. Factor Xa cleavage was stopped by adding 5mM benzaminide and 10 mM EDTA. Protein solution were concentrated using amicon pressure cells equipped with 10 kDa cutoff filters to 20 ml added to a 50 ml superloop before subjected to FPLC using a superloop and Pharmacia Sephadex G-75 column for 10 loops operated by the Äkta explorer system following the recommendations of the supplier. Protein fractions containing Mal d 1 were pooled added NaCl to 200 mM and DTT to 5 mM and filtered (45 µm) before subjected to a second round of amylose affinity chromatography. Protein Fractions containing purified Mal d 1 were pooled and dialyzed against 2x 5L of TRIS-HCl buffer pH 7.5 before concentrated using amicon pressure cells equipped with 3 or 10 kDa cutoff filters to >1 mg/ml for storage at 4°C or -80°C. Mal d 1 mutants with authentic amino-terminals were purified to electrophoretic homogeneity at 2-5 mg yields per liter of culture medium. All purified Mal d 1 preparations appeared as single bands after silver-stained SDS-polyacrylamide electrophoresis with an apparent molecular weight of 17.5 kDa.

Circular dichroism spectroscopy

Circular dichroism spectra were obtained using an OLIS DSM 10 CD spectrophotometer (On Line Instrument Systems, Bogart, GA, USA), equipped with cylindrical (31-Q-1/CD) or square (21-Q-1/CD) 0.1 cm light path quartz cuvettes (Starna, Essex, UK). The spectra were recorded from 260 nm to 184 nm collecting data at every second nm, 38 data points per spectrum. The temperature of the cuvette was maintained constant using a Julabo Model F30-C bath/circulator temperature control module (Julabo Labortechnik, Seelbach, Germany). Spectra were obtained in 0.01M sodium phosphate buffer, pH 7.2, at a concentration of 0.1 mg protein per mL. Each spectrum represents the arithmetic mean of four determinations corrected for

buffer absorption and normalized to $\Delta\epsilon = 0$ at 260 nm. The mDeg recorded was transformed to $\Delta\epsilon$ using:

$$\Delta\epsilon = \text{mDeg}/(32980 \cdot c \cdot l) \text{ Abs}_{\text{units}}/(M \cdot \text{cm})$$

Where mDeg is the circular dichroism signal, c is the concentration in mol/L as measured by amino acid analysis and l is the length of the light path in cm.

IgE-binding experiments

10 Sera from individual patients or a pool of equal volumes of sera from several birch pollen allergic patients were used for specific serum IgE inhibition assays. rBet v 1.2801 (database accession number: Z80104) expressed and purified as described for rMal d 1, was biotinylated at a molar ratio of 1:5 (Bet v 1:biotin). The inhibition assay was performed on ADVIA Centaur System (Bayer, Denmark) as follows: A serum sample (25 μl) was incubated with paramagnetic beads (solid phase) coated with a monoclonal mouse anti human IgE antibody (ALK-Abelló, Denmark), washed, resuspended and incubated with a mixture of biotinylated Bet v 1 and inhibitor (non-biotinylated Bet v 1, rMal d 1 (2620) or mutated rMal d 1 allergens) in dilution series. The amount of biotinylated Bet v 1 bound to the serum IgE on the solid phase was estimated from the measured relative light units (RLU) after incubation with acridiniumester labelled streptavidin. The degree of inhibition was calculated as the ratio between the RLU's obtained using buffer and mutant as inhibitor.

25 Histamine release experiments (rMal d 1 (2762) and (2781))

100 μl of pipes buffer pH 7.4 or 100 μl of allergen dilutions in Pipes buffer pH 7.4, 1 ng, 3 ng, 10 ng, 30 ng, 100 ng, 1000 ng of rBet v 1.2801, rMal d 1 (2620) or mutated rMal d 1 (2762) or (2781) were added to 96 well plates in triplicates. Plates were preheated to 37°C before 100 μl of preheated (37°C) 30 blood, diluted 1:5 with Pipes buffer pH 7.4, were added to wells with different allergen dilutions or Pipes buffer. Plates were then incubated 30 minutes at 37°C before centrifuged 10 minutes at 800g in a centrifuge with a plate-rotor. Supernatants from each of the wells were transferred to wells in new

microtiter-plates that were lid and sealed with sealing tape before incubated at 0°C.

Histamine release in each sample was measured using the ELISA based
5 Enzyme Immunoassay Kit ref. 2015 (Immunotech, France) following the recommendations of the supplier. 100 µl of sample or standard solutions were acylated with 25 µl of acylation buffer and 25 µl of acylation reagent to permit later binding to anti-histamine-antibodies. 50 µl of acylated histamine samples, standards or negative controls were then added to anti-histamine-
10 antibody coated wells along with 200 µl of alkaline phosphatase conjugated histamine and incubated 2 hours at 5°C with shacking. Plates were rinsed 3 times with diluted wash solution before 200 µl of Para-nitrophenylphosphate (pNPP) substrate solution was added to the plates that were incubated 30 minutes at room temperature with shacking before 50 µl of stop solution were
15 added. The ability of the histamine samples and histamine standards (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 1000 nM) to inhibit binding of alkaline phosphatase conjugated histamine to anti-histamine antibody coated wells were measured as relative absorbance at 405 nm.

20 **Results**

The polypeptide backbone absorbs and is optically active in the far UV region below 260 nm and the magnitude of the absorbance is depending upon conformation of the backbone. Measuring differences in absorption of left- and right-circularly polarized light circular dichroism spectroscopy is able to
25 detect alterations in secondary protein structure such as α -helices, β -sheets or random coil elements. In addition, measuring of circular dichroism spectra during protein heat-denaturing provide information about protein stability as it reveals spectra of native folded contra denatured proteins. To assess protein stability with respect to long term storage or buffer shifts circular dichroism spectroscopy was applied to all protein preparations prior to biological
30 testing. Heat denaturing was applied to rMal d 1 (2620). Non-mutated rMal d 1 (2620) and rMal d 1 mutants (2781 and 2762) all had circular dichroism (CD) spectra with comparable shape as the CD spectrum of native folded

~ rBet v 1.2801 (see figure 2). This shows that the rMal d 1 molecules contain the same secondary structure elements as rBet v 1.2801.

Isoforms of Mal d 1 from apple have from 50-60% amino acid identity with 5 Bet v 1 and due to this amino acid residue variation a degree of Bet v 1 specific cross-reactivity occurs. This is illustrated in figure 3A and 3B that show the binding of birch pollen allergic patients serum IgE, from two different serum pools to biotinylated rBet v 1.2801, inhibited by rMal d 1 (2620)[■]. The inhibition curves show that serum pool A is unable to bind 10 rMal d 1 (2620) and that rMal d 1 (2620) binds less than 20% of rBet v 1 specific IgE-antibodies in serum pool B. Being a naturally occurring isoform 15 rMal d 1 (2620) is a stable protein. This is illustrated in figure 4 where heat denaturing experiments show that rMal d 1 (2620) maintain most of its secondary structure elements at temperatures up to 70°C and by the spectrum of rMal d 1 obtained after heating to 90°C and cooled to 15°C that shows the ability of the protein to refold completely.

Isoforms of Mal d 1, e.g. rMal d 1 (2620) are thus regarded as good Bet v 1 scaffold candidates.

20 Despite some amino acid variation, one large surface area (see figure 5A and 5B) has a high degree of structural similarity between Mal d 1 and Bet v 1 proteins. Cross-reactivity in this area is to a great extent responsible for 25 apple induced oral allergy syndrome (OAS) in some birch pollen allergic patients. To reduce the potential of the scaffold protein rMal d 1 (2620) to trigger histamine release in birch pollen allergic patients three secondary mutations N28T, K32Q, E45S were introduced in surface areas on rMal d 1 (2620) common for birch and apple proteins (see figure 5C) This Mutant is 30 named rMal d 1 (2760). Other surface areas on rMal d 1 (2620) have a very high density of Mal d 1 specific residues not found in Bet v 1 and cross-reactivity is therefore not observed in these areas. To increase the potential of such surface structures to bind to Bet v 1 specific antibodies, specific amino acid residues were introduced into Mal d 1 specific surface areas on the scaffold protein. Two examples of rMal d 1 mutants that contain such

primary mutations are rMal d 1 (2781) (SEQ ID NO 2) (see figure 6A, B and C) and rMal d 1 (2762) (SEQ ID NO 3) (see figure 7A, B and C).

Primary mutations

5 In rMal d 1 (2762) and rMal d 1 (2781) introduction of 8 and 6 primary mutations, respectively, substantially increased the capacity of the mutants to bind birch pollen allergic patients serum IgE compared to rMal d 1 (2620). This is shown in figure 8A where the ability of rMal d 1 (2762) and rMal d 1 10 (2781) to inhibit binding of serum-IgE to biotinylated rBet v 1.2801 were increased from 0% to 62% and 39%, respectively, at inhibitor concentrations at 3000 ng/test. Still, compared to rBet v 1.2801 reaching 62% and 39% IgE inhibition with the mutants, respectively, required 500-fold and 1000-fold 15 higher antigen concentrations, respectively, showing that the rMal d 1 mutants still bind birch specific IgE with considerable lower affinities than rBet v 1.2801 protein.

20 The mutated rMal d 1 molecules were further tested in human basophile histamine release assays. No histamine release occurred with non-mutated rMal d 1 (2620) in the measured antigen concentration range. rBet v 1.2801 and mutated rMal d 1 all triggered histamine release. The histamine release curves of mutated rMal d 1 (2781) and (2762) are shifted 2-fold and 20-fold, respectively, toward higher antigen concentrations compared to rBet v 1.2801 as shown in figure 8B.

25 Secondary mutations

20 In figure 9 (right side) the scaffold protein rMal d 1 (2620) and mutated rMal d 1 (2760) inhibits binding of human serum IgE to rBet v 1.2801 from 0% to 30% in individual patients. rMal d 1 (2620) did show substantially reduced capacity to trigger histamine release compared to rBet v 1.2801 in some but 30 not all birch pollen allergic patients basophiles as shown in figure 9 (left side) Introduction of three secondary mutations Asn28Thr, Lys32Gln and Glu45Ser in Bet v 1 specific surface structures on the scaffold protein (rMal d 1 (2760) further reduced the capacity to trigger histamine in two birch pollen allergic patients basophiles (o, p) as shown in figure 9 (left side). Here introduction of

3 secondary mutations abolished histamine release in the measured antigen concentration range in patient (o) and 100-fold higher concentration of rMal d 1 (2760) was required to trigger histamine release in patient (p) compared to rBet v 1.2801.

5

Primary and secondary mutations

In figure 10 A and B a schematic illustration of a scaffold protein and a scaffold variant derived from the scaffold protein by introducing primary and secondary mutations is shown. rMal d 1 (2620) is used as a example. Front and back side of the proteins are illustrated. Scaffold (Mal d 1) specific surface structures are shown in white color. Bet v 1 specific surface structures are shown in grey color. Secondary mutations are shown in black color. Top front view shows two large surface areas on the scaffold protein that binds Bet v 1 specific human serum IgE with high affinity. Bottom front view show four secondary mutations introduced in the scaffold protein in order to reduce IgE-binding affinity. Top back view shows a surface area on the scaffold protein with few Bet v 1 specific surface structures. Bottom back view shows the result of introducing primary mutations to increase the density of Bet v 1 specific surface structures where against blocking antibodies cross-reactive with natural Bet v 1 isoallergens can be directed. Figure 10B. Top and bottom front views: A reduced fit of a Bet v 1 specific IgE antibody caused by introduction of 3 secondary mutations are illustrated. Top back view illustrates the generation of a blocking antibody (IgG) with pour antibody cross-reactivity to Bet v 1. Bottom back view illustrates the generation of a blocking antibody (IgG) with increased antibody cross-reactivity to Bet v 1

20 T-cell response to recombinant wt and mutated Mal d 1 allergens
Changes in the amino acid sequence of an allergen can potentially provoke the removal and/or addition of T-cell epitopes. In these cases, the modified allergens can provoke a reduced T-cell response compared to the response to the wild type allergen, and/or they will activate T-cells that recognize the new epitopes. Theoretically, it is of importance to maintain the T-cell stimulation properties of the Bet v 1 hypoallergenic Mal d 1 mutants to modify

the existing allergen-specific T-cell population and to support the production of blocking non-IgE antibodies. Therefore the T-cell activating properties of the rMal d 1 allergens were tested.

5 Two cell types were analysed in both cases cells were derived from birch allergic individuals:

1) Freshly isolated blood cells (PBL) containing many different T-cells. These T-cells have the ability to respond to Bet v 1-specific epitopes and Mal d 1-specific epitopes as well as new epitopes originating from changes in the sequence. The analysis was performed immediately after cell isolation. The blood cells were cultured for seven days in the presence of various concentrations of the allergen. PBL from three individual donors was tested.

15 2) Allergen-specific cell lines generated through repeated stimulation with nBet v 1. These cells can be kept in culture and tested over a long period of time. T-cells are cultured with the allergen for three days. Autologous PBL cells are added as antigen presenting cells (APC). These T-cells respond primarily to original Bet v 1-specific epitopes and show the level of T-cell activation within the existing Bet v 1-specific T-cell population of the allergic patient. Four Bet v 1-specific T-cell lines were tested.

The T-cell response to the rMal d 1 allergens has been analysed by means of two assays:

25 1) Stimulation of T-cell proliferation, measuring the incorporation of radioactive labelled thymidin into DNA during the last 18 hours of the culture.

30 2) Stimulation of cytokine production, measured in the cell supernatant by means of CBA, a FACS- based assay that measures 6 different Th1/Th2 cytokines.

Figure 11 and 12 show the results from PBL stimulation assays from three individual donors (fig 1) or in combination (fig 2). In these experiments the wt

Mal d 1 allergen (2620) and the triple Mal d 1 mutant (2760) do not induce any T-cell activation. The mutants 2762, in one case, and 2781, in three out of three PBL assays performed, induced T-cell proliferation comparable to the activation induced by rBet v 1 (2801), indicating that T-cell epitopes may

5 have been restored by the amino acid changes introduced in these molecules. Alternatively new epitopes may have been introduced leading to a comparable T-cell activation. As expected, the response to rBet v 1 is remarkably strong in these experiments.

10 Figure 13 shows the T-cell proliferative response of one of the four Bet v 1-specific T-cell lines tested. This T-cell line exhibit clear cross-reactivity to the wt Mal d 1 molecule (2620), but do not recognise the mutant 2760 suggesting that the mutations introduced in this molecule disrupt the Bet v 1/Mal d 1 cross-reacting T-cell epitope recognised by this T-cell line. In contrast, the 15 mutants 2762 and 2781 both seems to induce enhanced proliferation compared to the wt Mal d 1 molecule, again indicating that cross-reacting T-cell epitopes may have been restored.

20 The cytokine production in PBL cultures (figure 4) and in the cross-reactive T-cell line (figure 5) confirms the T-cell activation demonstrated in the proliferation assays. Significant changes in the cytokine production profiles were not observed when the response to Bet v 1, 2801 and the Mal d 1 allergens were compared, suggesting that the difference between Bet v 1 and Mal d 1 or the changes introduced through the mutations do not lead to 25 increased or selective Th1 activation. Induction of the inhibitory cytokine IL-10 was not observed.

30 In conclusion, the Mal d 1 mutants 2762 and especially 2781 seems to induce increased T-cell activation compared to the wt Mal d 1 allergen (2620) both within the Bet v 1-specific T-cell population and in freshly isolated blood cell cultures of allergic patients.

EXAMPLE 2

Modification of Bet v 1 homologous scaffold proteins (surface area I)

This example is based on modification of Dau c 1 (accession no.T14325).

5 Amino acid residue position numbering in the following refers to T14325 as shown in figure 16. The example is based on the introduction of one or more of the following secondary mutations: K32X, E42X, E59X, R69X, E95X, K122X where X can be any type of amino acid residue but preferably amino acid residues that are absent in corresponding positions of known isoforms of

10 Bet v 1 The example further includes the introduction of one or more of the following primary mutations where by introduced mutations are residues that are homologous more preferably identical to corresponding amino acid residues in any known isoforms of Bet v 1. Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that

15 two amino acid residues are homologous. From this the following mutations are suggested: S12V or S12L or S12I or S12M, S14P, E16A, P105A, A107P, A148S or A148T, I151L or I151V or I151M, N153H or N153K or N153R, and elongation of the amino acid sequence with one or more of the following amino acid residues +154S or +154T, +155D or +155E, +156A, +157Y or

20 +157F, +158N or +158Q. Schematic illustration of protein sequence with mutated amino acid residues are shown in figure 16.

Modification of Bet v 1 homologous scaffold proteins (surface area II)

This example is based on modification of Dau c 1 (accession no.T14325).

25 Amino acid residue position numbering in the following refers to T14325 as shown in figure 17. The example is based on the introduction of one or more of the following secondary mutations: E8X, T10X, D25X, K32X, D46X, E59X, E95X, D108X, K122X where X can be any type of amino acid residue but preferably amino acid residues that are absent in corresponding positions of

30 known isoforms of Bet v 1 The example further includes the introduction of one or more of the following primary mutations where by introduced mutations are residues that are homologous more preferably identical to corresponding amino acid residues in any known isoforms of Bet v 1. Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that two amino acid residues are homologous.

35

From this the following mutations are suggested: K39S or K39T, K44E or K44D, V52I or V52M or V52L, I54K or I54R or I54H, T64K or T64R or T64H, T65Y or T65F or T65W, T67K or T67R or T67H, D86E, L91G, G92D or G92E. Schematic illustration of protein sequence with mutated amino acid residues are shown in figure 17.

Modification of Der p 2 homologous scaffold proteins (surface area I)

This example is based on modification of Lep d 2 (accession no. S66499). Amino acid residue position numbering in the following refers to S66499 as shown in figure 18. The example is based on the introduction of one or more of the following secondary mutations: K6X, S22X, R30X, K76X, K81X, V114X where X can be any type of amino acid residue but preferably amino acid residues that are absent in corresponding positions of known isoforms of nDer p 2 or nDer f 2 or Eur m 2. The example further includes the introduction of one or more of the following primary mutations where by introduced mutations are residues that are homologous more preferably identical to corresponding amino acid residues in any known isoforms of nDer p 2 or nDer f 2 or nEur m 2. Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that two amino acid residues are homologous. From this the following mutations are suggested: D17L or D17I or D17V or D17M, S19P, Q32K or Q32R or Q32H, K33P, T35Q or T35N, N88K or N88R or N88H, T92N or T92Q, A95K or A95R or A95H. Schematic illustration of protein sequence with mutated amino acid residues are shown in figure 18. View of mutations in respect to tertiary protein structure are shown in figure 19 A, B, C and D and in figure 20 A, B, C and D.

Modification of Der p 2 homologous scaffold proteins (surface area II)

This example is based on modification of Lep d 2 (accession no. S66499). Amino acid residue position numbering in the following refers to S66499. The example is based on the introduction of one or more of the following secondary mutations: K6X, S22X, H29X, R30X, K76X, K81X where X can be any type of amino acid residue but preferably amino acid residues that are absent in corresponding positions of known isoforms of nDer p 2 or nDer f 2

or Eur m 2. The example further includes the introduction of one or more of the following primary mutations where by introduced mutations are residues that are homologous more preferably identical to corresponding amino acid residues in any known isoforms of nDer p 2 or nDer f 2 or nEur m 2.

5 Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that two amino acid residues are homologous). From this the following mutations are suggested: D45N or D45Q, N47K or N47R or N47H, K48T or K48S, T50K or T50R or T50H, K52E or K52D, L54K or L54R or L54H, E107K, E107R or E107H, H112D or H112E, T119I or 10 T119L or T119V or T119M. Schematic illustration of protein sequence with mutated amino acid residues are shown in figure 18, shown previously.

EXAMPLE 3

15 Modification of Bet v 1 homologous scaffold proteins

This example is based on modification of Mal d 1 (2620) (accession no. AJ488060). Amino acid residue position numbering in the following refers to AJ488060 as shown in figure 21. The example is based on the introduction of one or more of the following secondary mutations: E8X, N28X, K32X, E96X and amino acid insertion +X159 (X being any type of amino acid residue). In one preferable embodiment of the invention one or more of the introduced amino acid residues (X) may be amino acid residues that are homologous to Bet v 1 specific residues for each of the individually corresponding positions. Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that two amino acid residues are homologous. From this the following mutations are suggested: E8D, N28Q, K32R or K32H, E96D. The example further includes the introduction of one or more of the following primary mutations where introduced mutations are residues that are identical or homologous to corresponding amino acid residues in any known 20 isoforms of Bet v 1. From this the following mutations are suggested: E12V or E12I or E12M or E12L, H40S or H40T, E76H or E76R or E76K, E129A, P154S or P154T. Schematic illustration of protein sequence with mutated 30 amino acid residues are shown in figure 21.

Modification of Der p 2 homologous scaffold proteins

This example is based on modification of Gly d 2 (accession no. AJ272216). Amino acid residue position numbering in the following refers to AJ272216 as shown in figure 22. The example is based on the introduction of one or more 5 of the following secondary mutations: K6X, R30X, F74X, K81X, K88X, T90X, V114X (X being any type of amino acid residue). In one preferable embodiment of the invention one or more of the introduced amino acid residues (X) may be amino acid residues that are homologous to Der p 2 or Der f 2 or Eur m 2 specific residues for each of the individually corresponding 10 positions. Homologous amino acid residues are: E=D, V=L=I=M, S=T, Y=F=W, K=R=H, N=Q, where (=) indicates that two amino acid residues are homologous. From this the following mutations are suggested: K6R or K6H, R30K or R30H, F74Y or F74W, K81R or K81H, K88R or K88H, T91S, V114L or V114I or V114M. The example further includes the introduction of one or 15 more of the following primary mutations where by introduced mutations are residues that are identical or homologous to corresponding amino acid residues in any known isoforms of Der p 2 or Der f 2 or Eur m 2. From this the following mutations are suggested: K2Q or K2N, K4D or K4E, K10N or K10Q, T14K OR T14R, S22H or S22R or S22K, K39V or K39L or K39I or 20 K39M, D45N or D45Q, T60L or T60V or T60I or T60M, Q63D, or Q63E, K80V or K80L or K80I or K80M, H112D or H112E, R122I or R122V or R122L or R122M. Schematic illustration of protein sequence with mutated amino acid residues are shown in figure 22. View of mutations in respect to tertiary protein structure are shown in figure 23 A, B, C and D and in figure 24 A, B, 25 C and D.

EXAMPLE 4

5 Comparison of secondary structure elements in homogeneous preparations of homogeneously folded proteins by CD spectroscopy or an experimental approach to determine if the three dimensional structures of two proteins share significant homologies:

10 Visual comparison of CD spectra determined for different or homologous proteins may be performed by an expert in the field. If the spectra seem to be reasonably super imposable one may assume that the proteins probably belong to the same structural class and a deconvolution process may confirm this classification.

15 CD spectra recorded in the wavelength range 260 nm to at least 184 nm were deconvoluted by the freeware program CDNN:

www.bioinformatik.biochemtech.uni-halle.de/cdnn

20 The deconvolution was performed as follows:

25 CD data (as a comma delimited txt file (nm;mDeg)) were loaded into the software and appropriate parameters were provided (Mw, concentration (mg/ml), number of amino acids) and the spectra was deconvoluted using a training base spectra set of 33.

30 The concentration of the protein was adjusted to a value giving a total of secondary structure elements of 100 % \pm 1% (185-260nm).

35 The secondary structure elements were transformed into three values Helix, "Sheet" = anti-parallel + parallel and Turn + coil = Beta-turn + random coil. The proteins were considered to be structurally similar if their deconvolution set (Helix, Sheet, Turn+coil) were within the values of the deconvolution set determined for the reference protein \pm 20%.

According to Fig. 25, The deconvolution sets of Mal d 1 (2762), Mal d 1 (2781) and Bet v 1.2801 fall within the acceptance boundaries determined for the reference protein, Mal d 1 (2620) ($\pm 15\%$), and therefore these proteins may be considered to be structurally (secondary structure) similar.

CLAIMS

1. A recombinant protein variant with the ability to induce a protective immune response to a naturally occurring allergen, wherein the protein
5 variant
 - is a variant of a scaffold protein, said scaffold protein has a three-dimensional folding pattern that is structurally similar to that of the naturally occurring allergen,
 - 10 • compared to the scaffold protein, comprises two or more primary mutations spaced by at least one non-mutated amino acid residue, each primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to the amino acid residue or residues in corresponding position the naturally occurring allergen,
 - 15 • and
 - has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.
2. A protein variant according to any of the preceding claims 1, wherein the protein variant has a reduced ability to induce histamine release compared to the naturally occurring allergen.
20
3. A protein variant according to claim 2 wherein the ability to induce histamine release is reduced 2 - 10.000 fold.
25
4. A protein variant according to any of the preceding claims, which further comprises one or more secondary mutations introducing into the scaffold protein amino acid residues, which are not present in the corresponding position in the natural occurring allergen.
- 30 5. A protein variant according to any of the preceding claims comprising 2-50, preferably 2-40, more preferably 3-25, more preferably 4-15 and most preferably 5-12 primary mutations.

6. A protein variant according to any of the preceding claims, wherein the scaffold protein has a level of amino acid identity with the naturally occurring allergen of between 20 and 60 %, preferably between 30 and 50 %.
- 5
7. A protein variant according to any of the preceding claims, wherein the protein variant compared to the naturally occurring allergen has a decreased binding capacity with respect to antibodies specific to the naturally occurring allergen.
- 10
8. A protein variant according to claim 1-7, wherein the said binding capacity is increased to at least 10 %, preferably to at least 50 %, and up to 100 % of the antibody binding capacity of the natural allergen.
- 15
9. A protein variant according to any of the preceding claims, wherein at least one of the primary mutations is a substitution.
10. A protein variant according to any of the preceding claims, wherein the introduction of at least one of the primary mutations is a deletion and/or an addition.
- 20
11. A protein variant according to any of the preceding claims, wherein the deconvoluted CD-spectra of the protein variant deviates less than 30%, preferably less than 20%, and even more preferably less than 10% compared to the deconvoluted CD-spectra of the naturally occurring allergen.
- 25
12. A protein variant according to any of the preceding claims, wherein all primary mutations are located within a surface region having an area of about 600-900 A².
- 30
13. A protein variant according to any of the preceding claims, wherein the primary mutations comprise mutation of surface-exposed amino acids.

14. A protein variant according to claim 13, wherein the primary amino acid residues to be mutated have a solvent accessibility of above 20 %, preferably above 30 %, more preferably above 40 %, and most preferably above 50 %.

5 15. A protein variant according to any of the preceding claims, wherein one or more of the mutations are carried out by site-directed mutagenesis.

16. A protein variant according to any of the preceding claims, wherein one or more of the mutations are carried out by DNA shuffling.

10 17. A protein variant according to any of the preceding claim obtained by gene library methods.

18. A method of producing a recombinant protein variant with the ability to induce a protective immune response to a naturally occurring allergen, comprising the steps of:

15

20

25

- selecting a scaffold protein, said scaffold protein having a three-dimensional folding pattern that is structurally similar to that of the naturally occurring allergen,
- introducing two or more primary mutations, that are spaced by at least one non-mutated amino acid residue, into the scaffold protein, each primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to the corresponding amino acid residue or residues in the naturally occurring allergen, and
- the protein variant having an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.

30 19. A product obtainable by the process of claim 18.

20. A protein variant according to any of the preceding claims, wherein the naturally occurring allergen is an inhalation allergen.

21. A protein variant according to claim 20, wherein the naturally occurring allergen is a pollen allergen.
22. A protein variant according to claim 21, wherein the naturally occurring allergen is a pollen allergen originating from the taxonomic order of *Fagales*, *Oleales* or *Pinales*.
23. A protein variant according to claim 22, wherein the naturally occurring allergen is *Bet v 1*.
24. A protein variant according to claim 23, wherein the scaffold protein is *Mal d 1*.
25. A protein variant according to claim 24, wherein at least two primary mutations are selected from the group consisting of: (E12V, E12I, E12M, E12L), P16A, (H40S, H40T), I43N, L44I, D47N, G65K, K70R, (E76H, E76R, E76K, Q76H), S107T, G108P, +109D, S110G, E129A, K152L, (P154S, P154T), P155S and optionally one or more secondary mutations are selected from the group consisting of: N28X, preferably N28T, K32X, preferably K32Q, E45S, E96X, +159X.
26. A protein variant (r*Mal d 1* (2781)) according to claim 24 comprising the sequence defined in SEQ ID NO 2, said variant comprising the following primary mutations: I43N, L44I, D47N, G65K, K70R, Q76H.
27. A protein variant (r*Mal d 1* (2762)) according to claim 24 comprising the sequence as defined in SEQ ID NO 3, said variant comprising the following mutations: E12V, P16A, K152L, P155S, S107T, G108P, +109D, S110G.
28. A protein variant according to claim 24 that comprise at least two primary mutations selected from the group consisting of: (E12V, E12I, E12M, E12L), (H40S, H40T), (E76H, E76R, E76K), E129A, (P154S, P154T), and optionally one or more secondary mutations selected from the group consisting of: E8X, N28X, K32X, E96X, +159X.

29. A protein variant according to claim 23 wherein the scaffold protein of Bet v 1 is Dau c 1.

5 30. A protein variant according to claim 29, wherein at least two primary mutations are selected from the group consisting of: (S12V, S12L, S12I, S12M), S14P, E16A, P105A, A107P, (A148S, A148T), (I151L, I151V, I151M), (N153H, N153K, N153R), (+154S, +154T), (+155D, +155E), +156A, (+157Y, +157F), (+158N, +158Q), (K39S, K39T), (K44E, K44D), (V52I, V52M, V52L), (I54K, I54R, I54H), (T64K, T64R, T64H), (T65Y, T65F, T65W), (T67K, T67R, T67H), D86E, L91G, (G92D, G92E) and optionally one or more secondary mutations are selected from the group consisting of: K32X, E42X, E59X, R69X, E95X, K122X, E8X, T10X, D25X, K32X, D46X, E59X, E95X, D108X, K122X.

15 31. A protein variant according to claim 29 that comprises at least two primary mutations selected from the group consisting of: (S12V, S12L, S12I, S12M), S14P, E16A, P105A, A107P, (A148S, A148T), (I151L, I151V, I151M), (N153H, N153K, N153R), (+154S, +154T), (+155D, +155E), +156A, (+157Y, +157F), (+158N, +158Q) and optionally one or more secondary mutations selected from the groups consisting of: K32X, E42X, E59X, R69X, E95X, K122X.

25 32. A protein variant according to claim 29 that comprises at least two primary mutations selected from the group consisting of: (K39S, K39T), (K44E, K44D), (V52I, V52M, V52L), (I54K, I54R, I54H), (T64K, T64R, T64H), (T65Y, T65F, T65W), (T67K, T67R, T67H), D86E, L91G, (G92D, G92E) and optionally at least one secondary mutation is selected from the group consisting of: E8X, T10X, D25X, K32X, D46X, E59X, E95X, D108X, K122X.

30 33. A protein variant according to claim 20, wherein the naturally occurring allergen originates from the taxonomic order of *Poales*.

34. A protein variant according to claim 20, wherein the naturally occurring allergen originates from the taxonomic order of *Asterales* or *Urticales*.

35. A protein variant according to claim 20, wherein the naturally occurring allergen is a house dust mite allergen.

36. A protein variant according to claim 35, wherein the naturally occurring allergen is a mite allergen originating from *Dermatophagoides*.

10 37. A protein variant according to claim 36, wherein the naturally occurring allergen is Der p 2.

38. A protein variant according to claim 37, wherein the scaffold protein is Eur m 1.

15 39. A protein variant according to claim 37, wherein the scaffold protein is Lep d 2.

40. A protein variant according to claim 39 that comprises at least two primary mutations selected from the group consisting of: D17L, D17I, D17V, D17M, S19P, Q32K, Q32R, Q32H, K33P, T35Q, T35N, N88K, N88R, N88H, T92N, T92Q, A95K, A95R, A95H and optionally one or more secondary mutations selected from the group consisting of: K6X, S22X, R30X, K76S, K81X, V114X.

25 41. A protein variant according to claim 39 that comprises at least two primary mutations selected from the group consisting of: D45N, D45Q, N47K, N47R, N47H, K48T, K48S, T50K, T50R, T50H, K52E, K52D, L54K, L54R, L54H, E107K, E107R, E107M, H112D, H112E, T119I, T119L, T119V, T199M and optionally one or more secondary mutations selected from the group consisting of: K6X, S22X, K29X, R30X, K76X, K81X.

30 42. A protein variant according to claim 37 wherein the scaffold protein is Gly d 2.

43. A protein variant according to claim 42 that comprises at least two primary mutations selected from the group consisting of: K2Q, K2N, K4D, K4E, K10N, K10Q, T14K, T14R, S22H, S22R, S22K, K39V, K39L, K39I, 5 K39M, D45N, D45Q, T60L, T60V, T60I, T60M, Q63D, Q63E, K80V, K80L, K80I, K80M, T91S, H112D, H112E, R122I, R122V, R122L, R122M and optionally one or more secondary mutations selected from the group consisting of: K6X, preferably K6R or K6H, R30X, preferably R30K or R30H, F74X, preferably F74Y or, F74W, K81X, preferably K81R or K81H, K88X, 10 preferably K88R or K88H, T90X, and V114X, preferably V114L, V114I or V114M.

44. A protein variant according to claim 20, wherein the naturally occurring allergen is a cockroach allergen. 15

45. A protein variant according to claim 19, wherein the naturally occurring allergen is an animal allergen, preferably a mammalian allergen.

46. A protein variant according to claim 45, wherein the naturally occurring 20 allergen is an allergen originating from cat, dog or horse.

47. A protein variant according to any of claims 1-19, wherein the naturally occurring allergen is a venom allergen.

25 48. A protein variant according to claim 47, wherein the naturally occurring allergen originates from the taxonomic order of *Hymenoptera*.

49. A protein variant according to claim 48, wherein the naturally occurring 30 allergen originates from the taxonomic orders of *Vespidae*, *Apidae*, or *Formicidae*.

50. A protein variant according to claim 49, wherein the naturally occurring allergen is *Ves v 5*.

51. A protein variant according to any of claims 1-19, wherein the naturally occurring allergen is a food allergen.

52. A recombinant protein variant with the ability to modulate an immune response to a naturally occurring allergen wherein

- the naturally occurring allergen is selected from the group consisting of plant, grass, food, and mite allergens,
- the protein variant is a variant of a scaffold protein, said scaffold protein has a three-dimensional folding pattern that is similar to that of a naturally occurring allergen, the protein variant compared to the scaffold protein comprises at least one primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to at least one amino acid residue present in the corresponding position in the natural occurring allergen, and
- the protein variant has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.

53. A recombinant protein variant with the ability to modulate an immune response to a naturally occurring allergen wherein

- the protein variant is a variant of a scaffold protein, said scaffold protein has a three-dimensional folding pattern that is similar to that of the naturally occurring allergen,
- the scaffold protein having a level of sequence identity with the naturally occurring allergen of between 30 and 50 %,
- the protein variant compared to the scaffold protein comprises at least one primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to at least one amino acid residue present in the corresponding position in the natural occurring allergen, and
- the protein variant has an increased allergen specific IgE affinity and/or binding capacity compared to the scaffold protein.

54. A recombinant protein variant with the ability to modulate an immune response to a naturally occurring allergen, wherein

- the protein variant is a variant of a scaffold protein, said scaffold protein has a three-dimensional folding pattern that is similar to that of a naturally occurring allergen,
- the deconvoluted CD-spectra of the protein variant deviates less than 5 30%, preferably less than 20%, and even more preferably less than 10% compared to the deconvoluted CD-spectra of the naturally occurring allergen,
- the protein variant compared to the scaffold protein comprises at least 10 one primary mutation introducing into the scaffold protein at least one amino acid residue identical or homologous to at least one amino acid residue present in the corresponding position in the natural occurring allergen, and
- the protein variant has an increased allergen specific IgE affinity 15 and/or binding capacity compared to the scaffold protein.

55. A protein variant according to any of claims 1-54 for use as a pharmaceutical.

56. Use of a recombinant protein variant according to any of claims 1-54 for 20 the manufacture of a medicament for the treatment or prevention of allergy.

57. A composition comprising two or more recombinant protein variants according to any of claims 1-54, wherein each variant is defined by having at 25 least one primary mutation, which is absent in at least one of the other variants.

58. A composition according to claim 57 comprising 2-12, preferably 3-10, more preferably 4-8, and most preferably 5-7 different protein variants according to claim 1-21.

30 59. A composition according to claim 57 or 58 for use as a pharmaceutical.

60. Use of a composition according to claim 57-59 for preparing a pharmaceutical for preventing and/or treating allergy.

61. A pharmaceutical composition, characterised in that it comprises a recombinant protein variant according to any of claims 1-54 or a composition according to claim 57 or 58.

5 62. A pharmaceutical composition according to claim 57 or 58 further comprising a pharmaceutically acceptable carrier and/or excipient and/or an adjuvant.

10 63. A pharmaceutical composition according to claim 57 or 58, wherein it is in the form of a vaccine against allergic reactions elicited by a naturally occurring allergen in patients suffering from allergy.

15 64. A method of generating an immune response in a subject comprising administering to the subject a recombinant protein variant according to any of claims 1-54, a composition according to claim 57 or 58, or a pharmaceutical composition according to any of claims 61-63.

20 65. A method of vaccination or treatment of a subject comprising administering to the subject a recombinant protein variant according to any of claims 1-54, a composition according to claim 57 or 58, or a pharmaceutical composition according to any of claims 61-63.

25 66. A process for preparing a pharmaceutical composition according to claim 57 or 58 comprising mixing a recombinant protein variant according to any of claims 1-54 or a composition according to claim 57 or 58 with pharmaceutically acceptable substances and/or excipients and/or adjuvants.

30 67. A pharmaceutical composition obtainable by the process according to claim 66.

68. A method for the treatment, prevention or alleviation of allergic reactions in a subject comprising administering to a subject a recombinant protein

variant according to any of claims 1-54, a composition according to claim 57 or 58, or a pharmaceutical composition according to any of claims 61-63.

69. A method of preparing a recombinant protein variant according to any of 5 claims 1-54, wherein the recombinant protein variant is produced from a DNA sequence obtained by DNA shuffling (molecular breeding) of DNA encoding the scaffold protein and the naturally occurring allergen.

70. A DNA sequence encoding a recombinant protein variant according to 10 any of claims 1-54.

71. A DNA sequence according to claim 70, wherein the derivative is obtained by site-directed mutagenesis of the DNA encoding the scaffold protein.

15 72. A DNA sequence according to any of claims 70-71, wherein the sequence is a derivative of a sequence selected from the group consisting of: SEQ ID NOs 2 and 3.

20 73. An expression vector comprising the DNA according to any of claims 70-72.

74. A host T-cell comprising the expression vector of claim 73.

25 75. A method of producing a recombinant mutant protein variant comprising the step of cultivating the host T-cell according to claim 74.

76. A recombinant protein variant according to any of claims 1-54 comprising at least one T-cell epitope capable of stimulating a T-cell clone or T-cell line 30 specific for the naturally occurring allergen.

77. A diagnostic assay for assessing relevance, safety, or outcome of therapy of a subject using a recombinant protein variant according to any of claims 1-54 or a composition according to claim 57 or 58, wherein an IgE

containing sample of the subject is mixed with said protein variant or said composition and assessed for the level of reactivity between the IgE in said sample and said protein variant.

SUMMARY

The present invention relates to novel recombinant protein variants that are useful as immunotherapeutic components. Also the present invention relates 5 to DNA sequences encoding said protein variants as well as compositions comprising said protein variants.

Modtaget

01 NOV. 2002

PVS

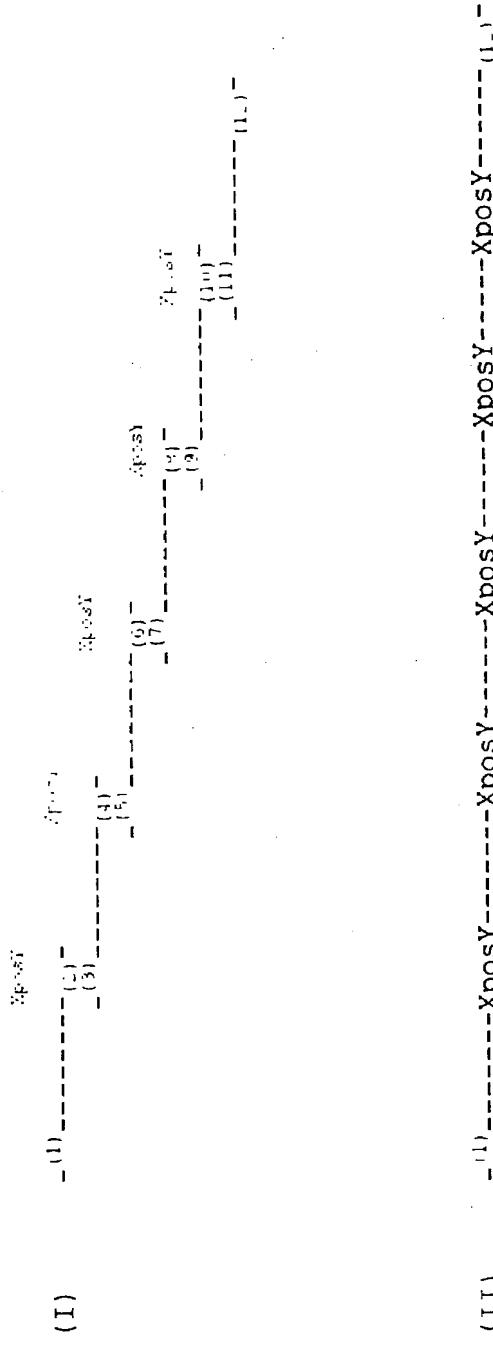


Figure 1

Modtaget
01 NOV. 2002
PVS

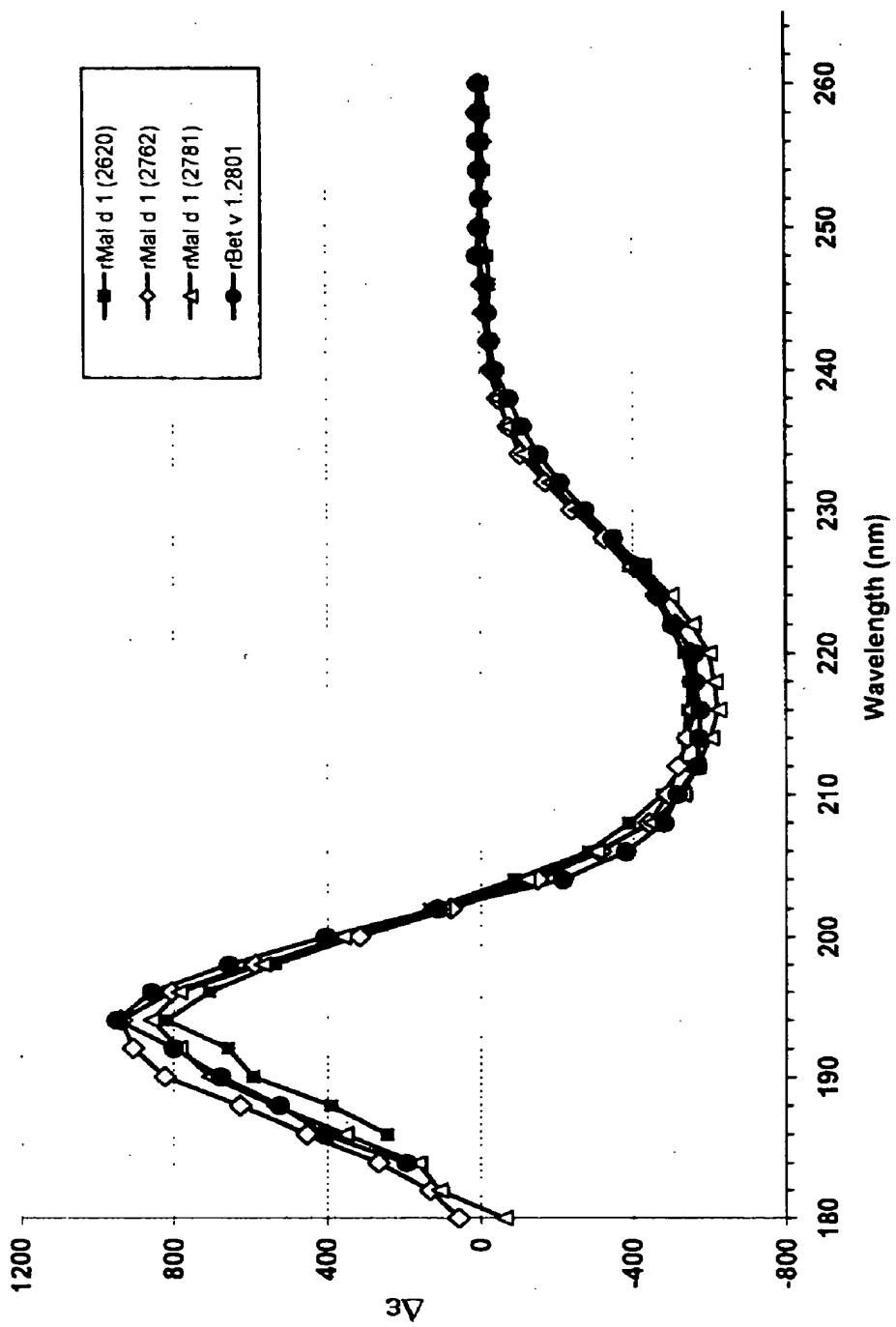


Figure 2

Modtaget

01 NOV. 2002

PVS

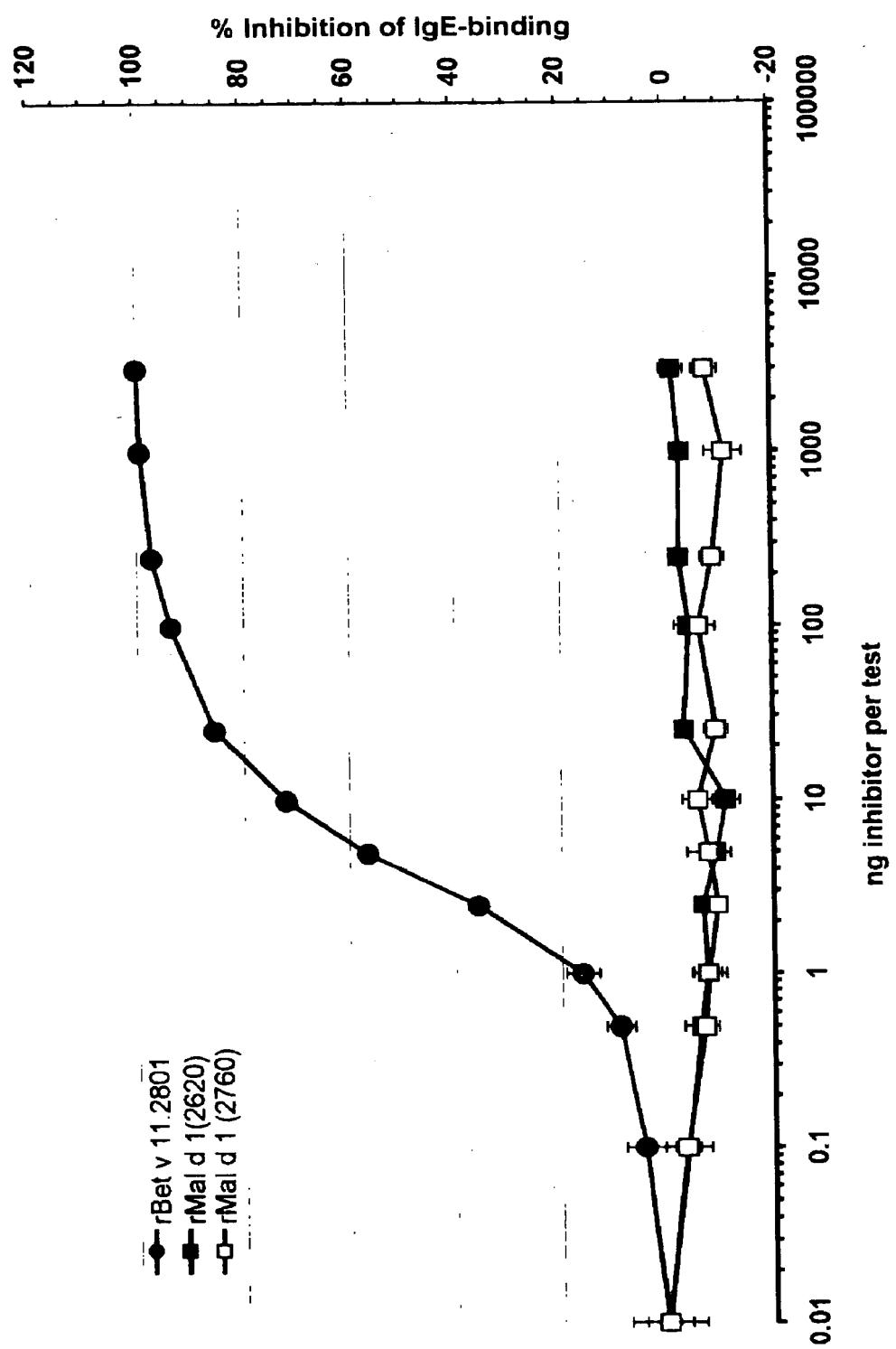


Figure 3A

Modtaget
01 NOV 2002
PVS

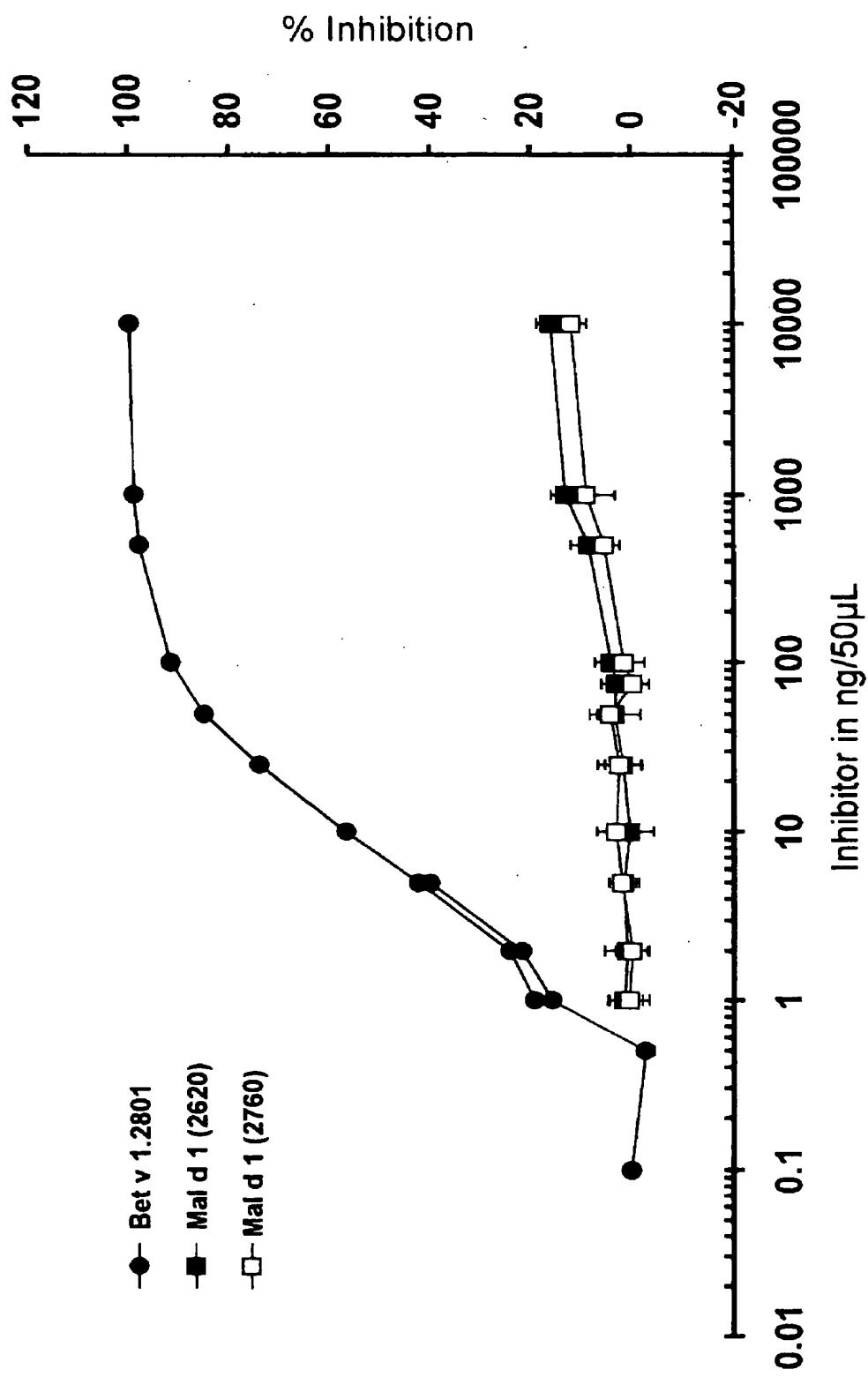


Figure 3B

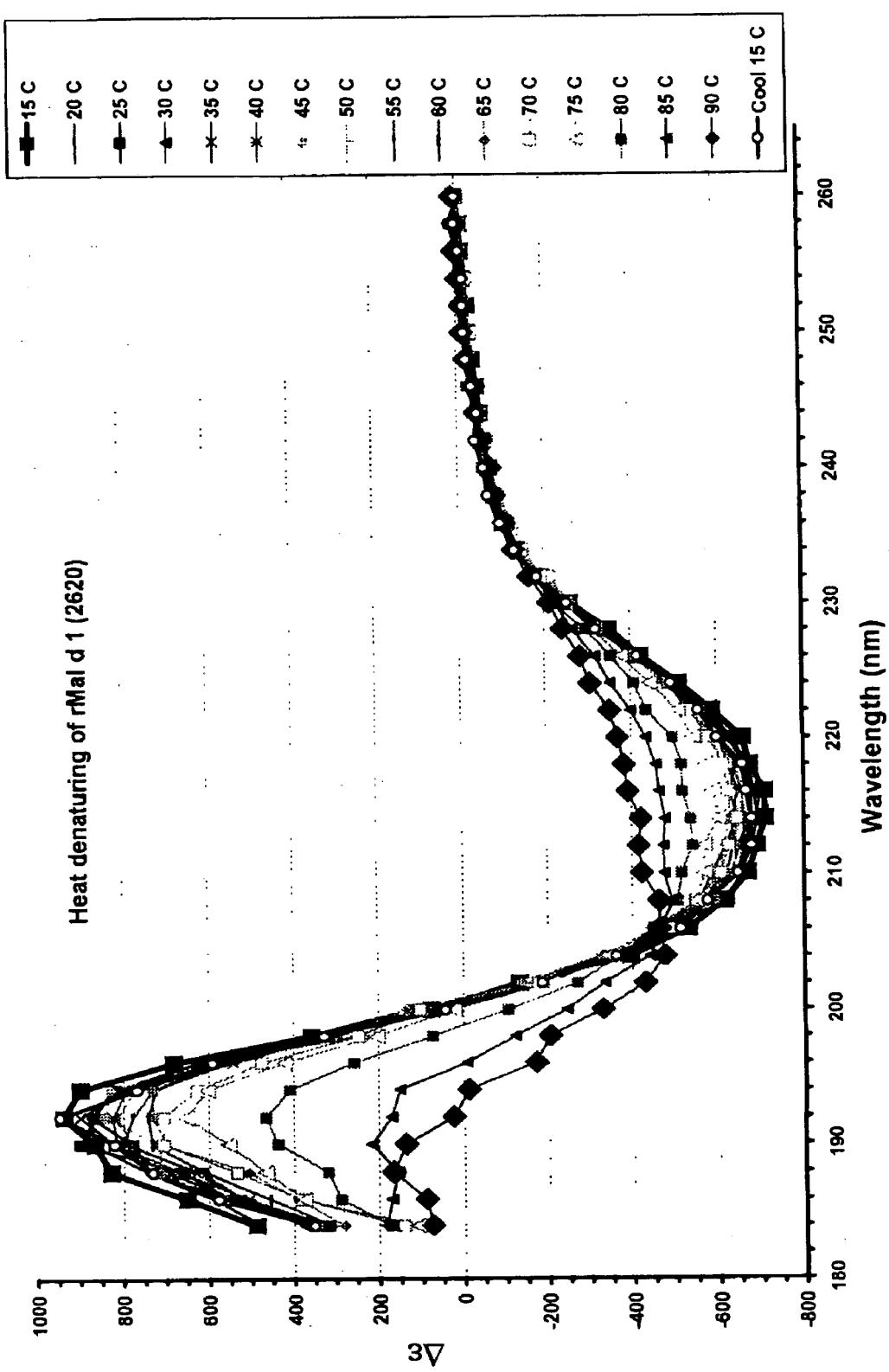


Figure 4

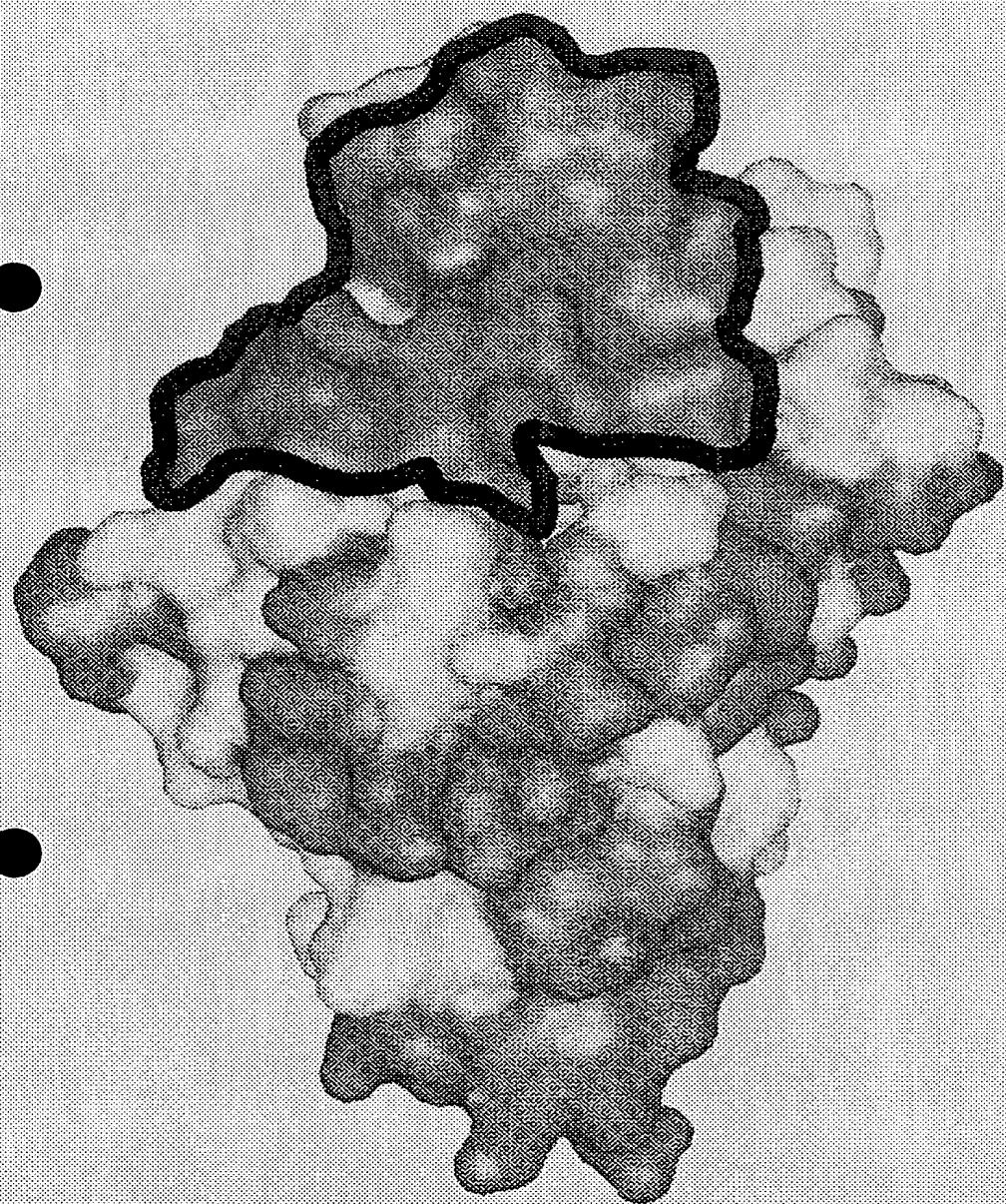


Figure S4

Madagascar

91 NOV. 2002

PVS

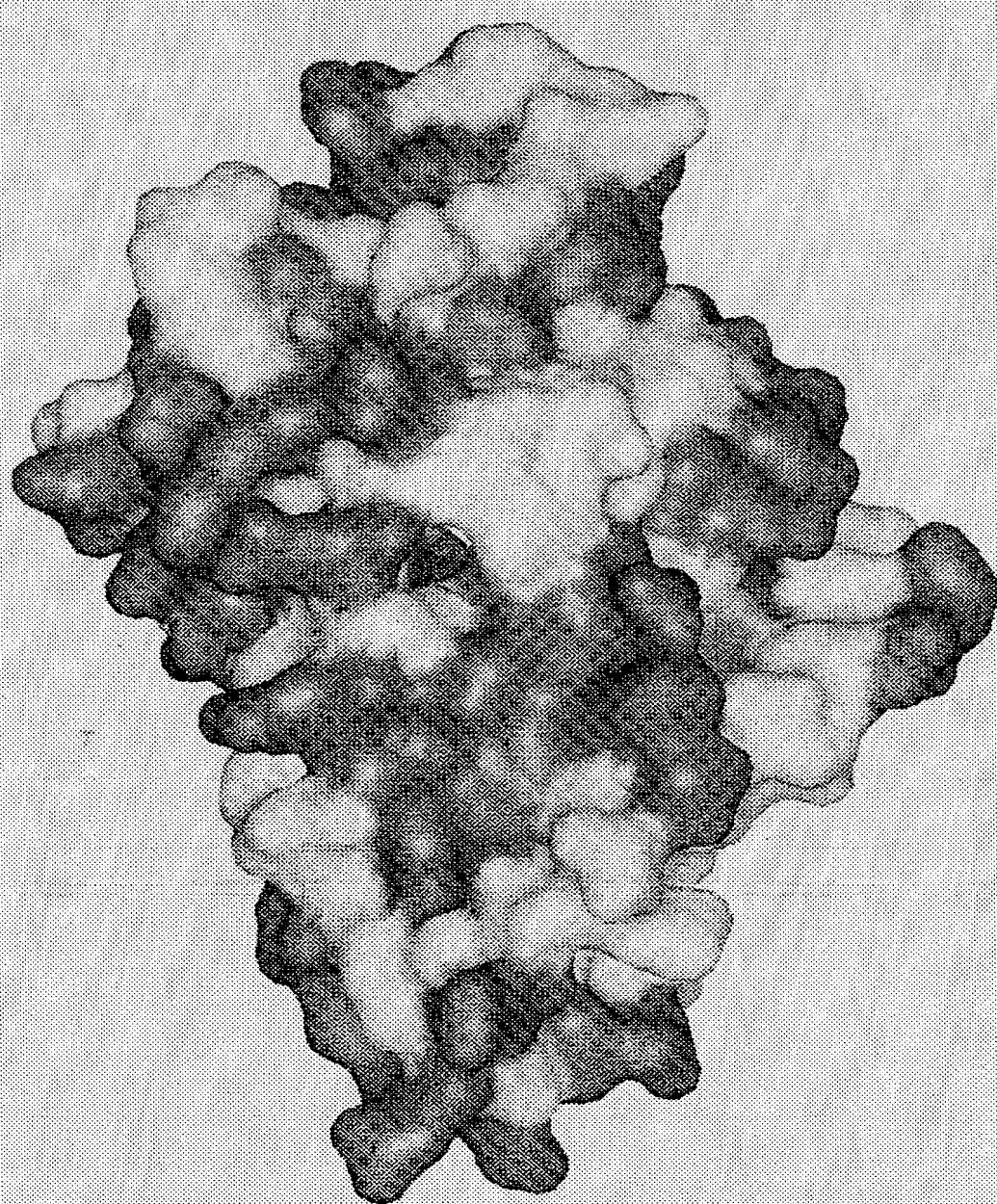


Figure 5B

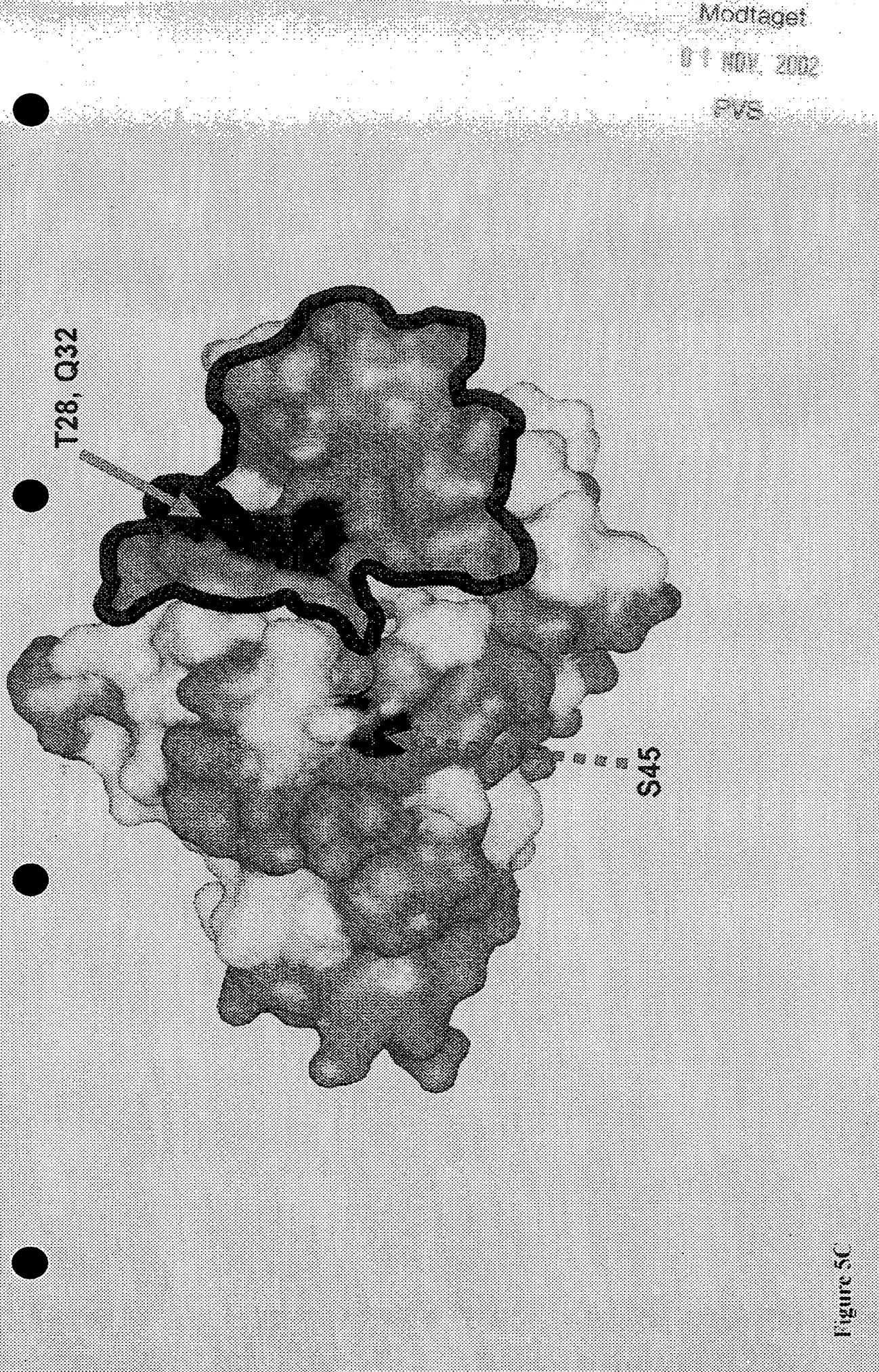


Figure 5C

Modtaget

01 NOV. 2002

PVS

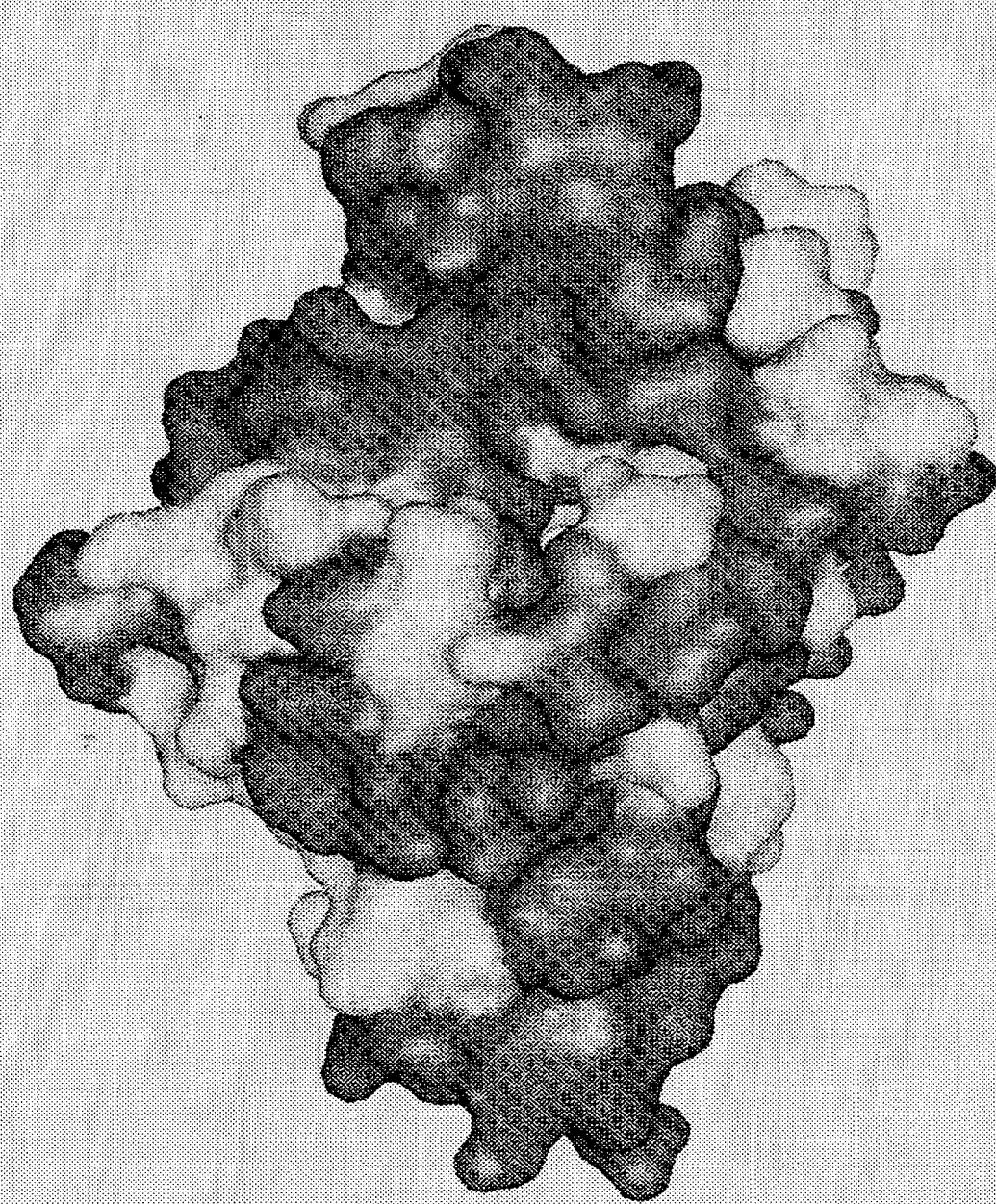


Figure 6A

Modtaget

01.09.2002

FYS

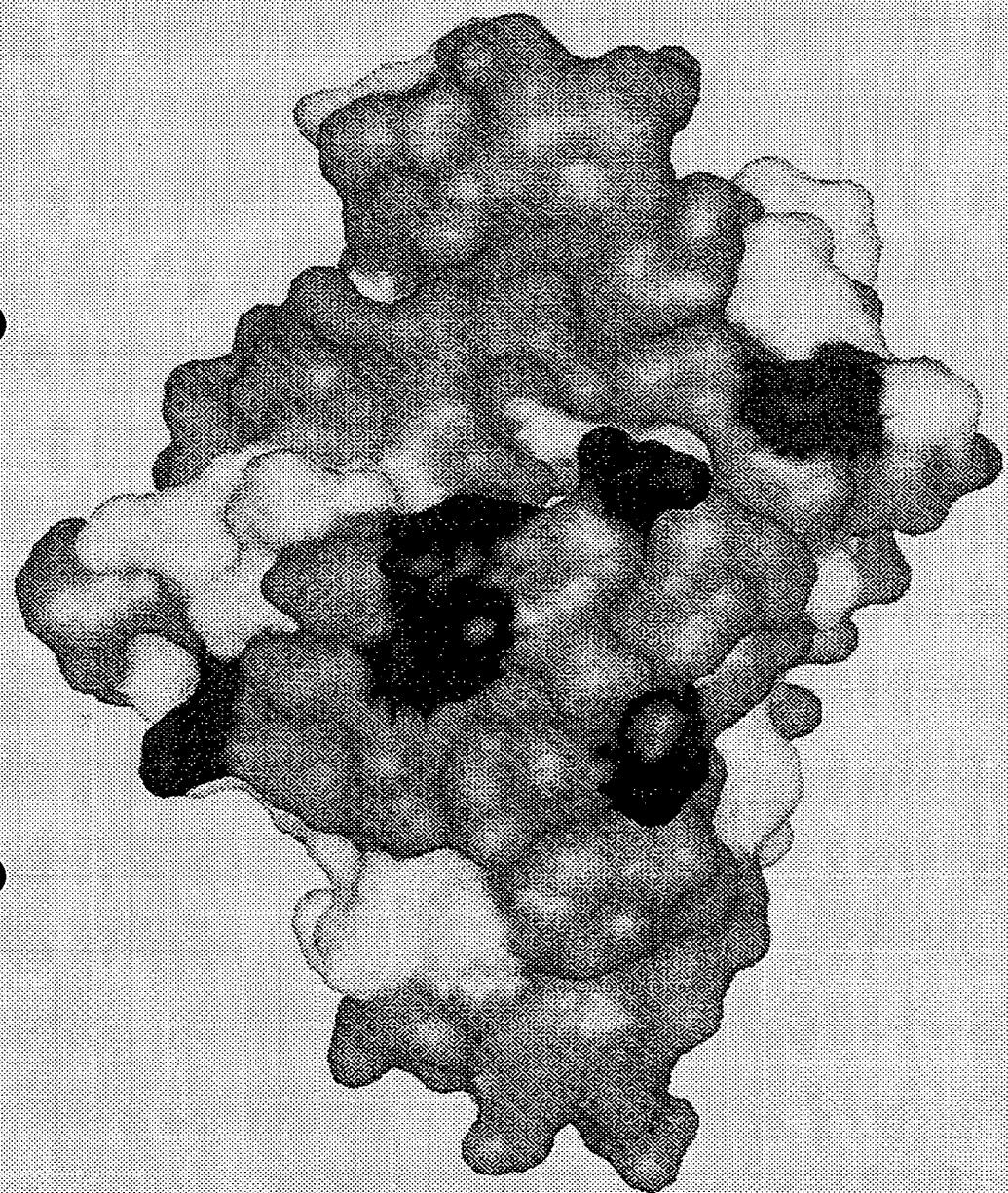


Figure 611

Modtaget

9-1 NOV 2002

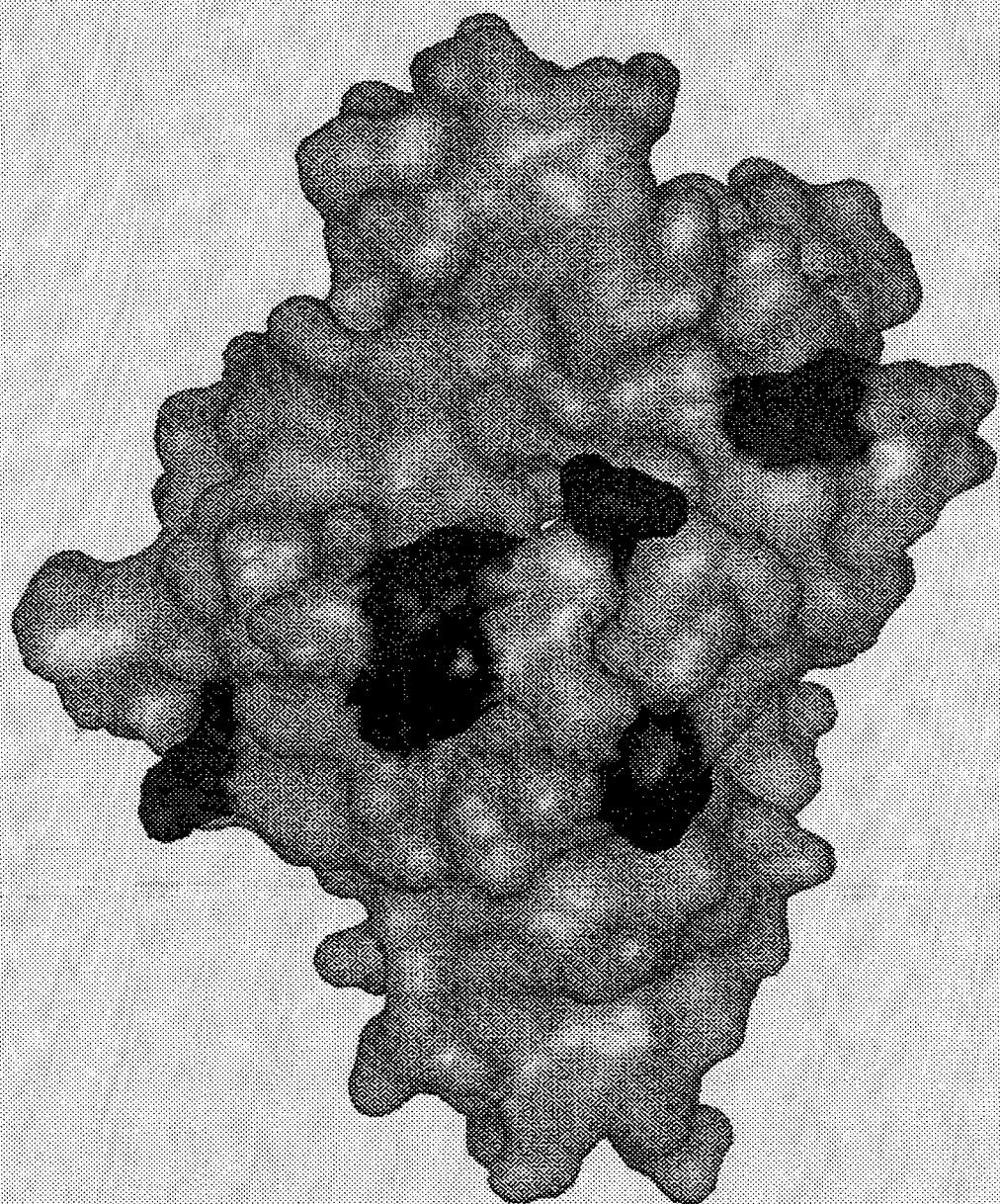


Figure 6C

Modtaget

01 NOV. 2002

PVS

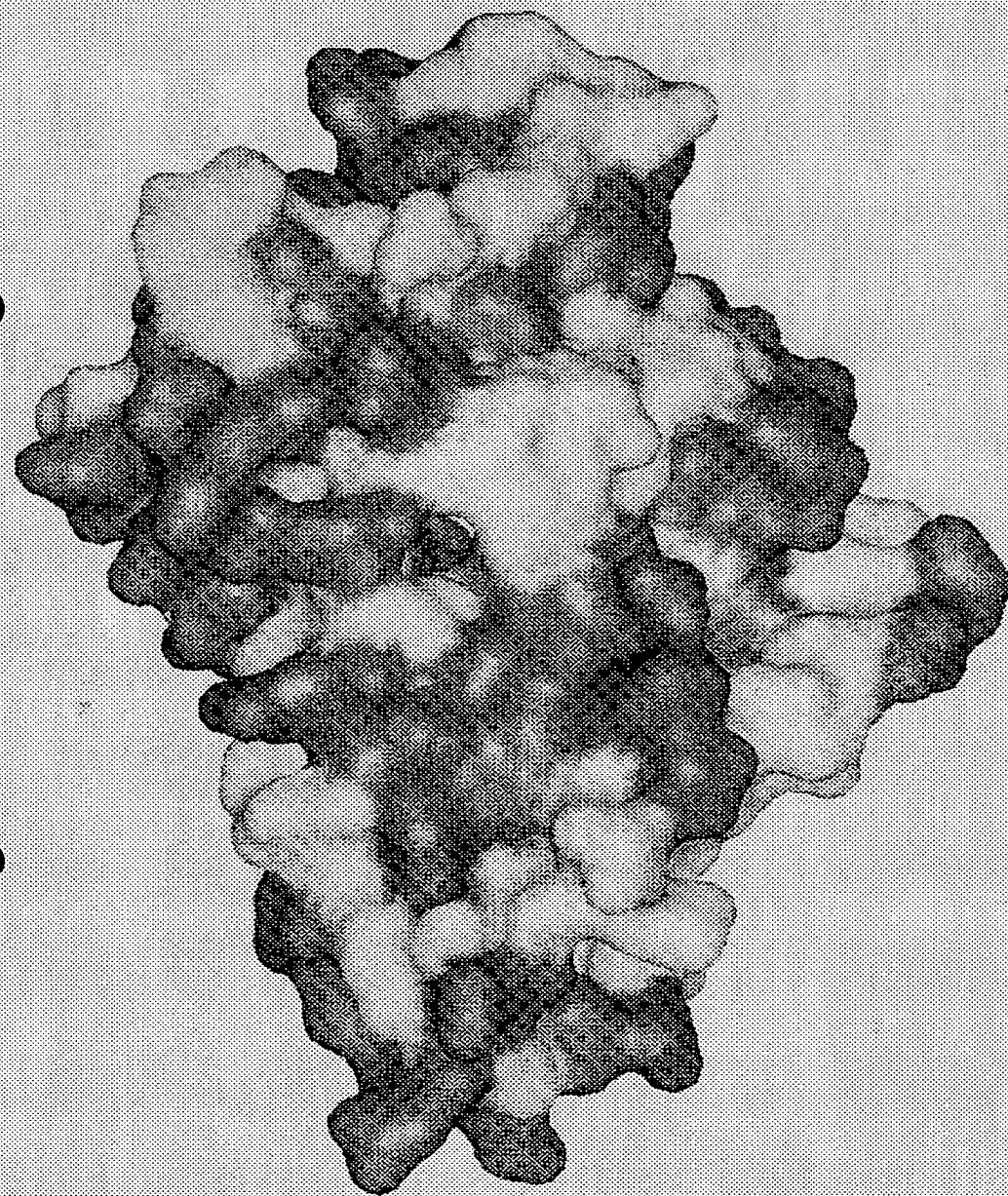


Figure 7A

Modtaget

01. NOV. 2004

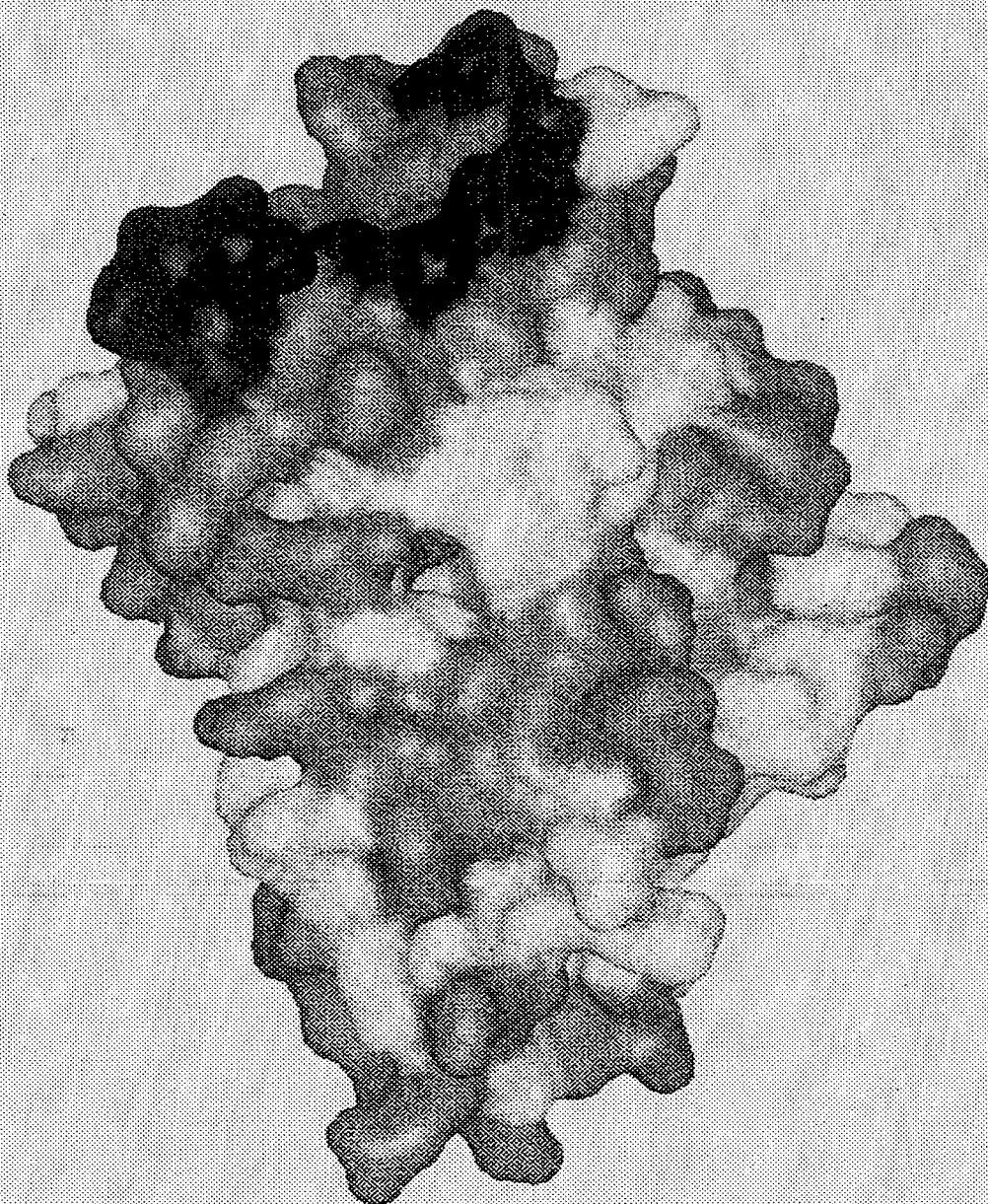


Figure 7B

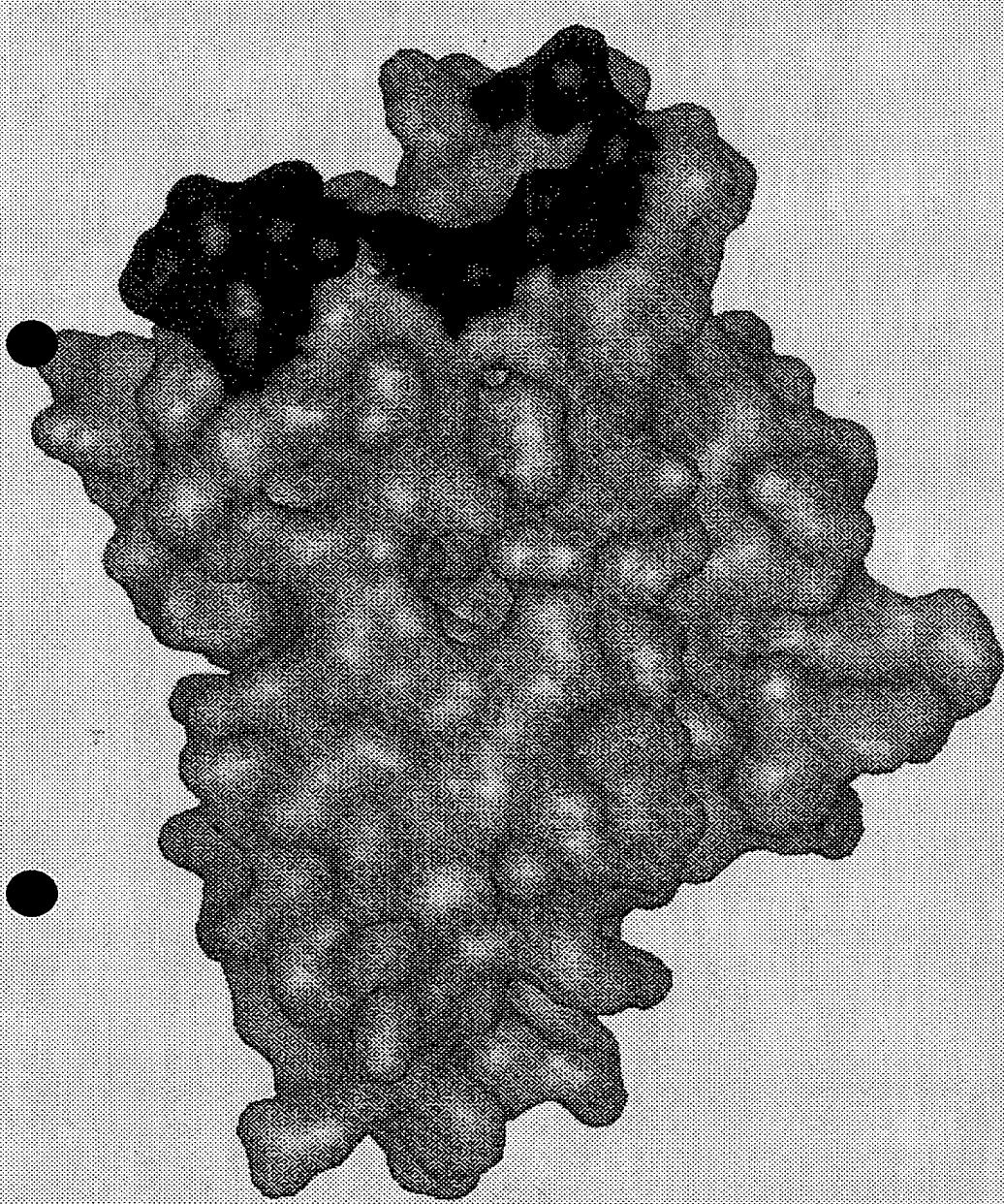


Figure 7C

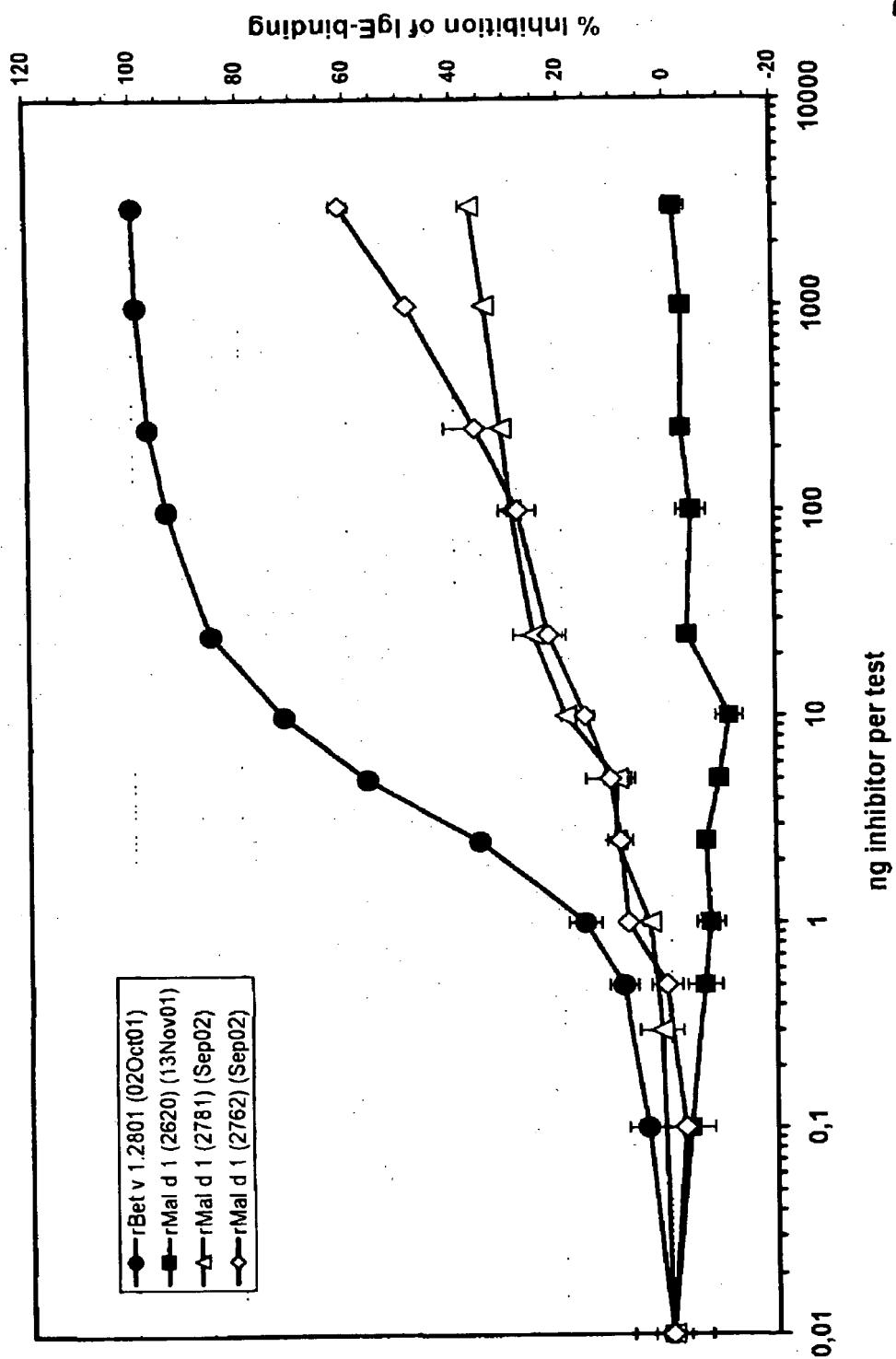


Figure 8A

Modtaget
07 NOV. 2002
PVS

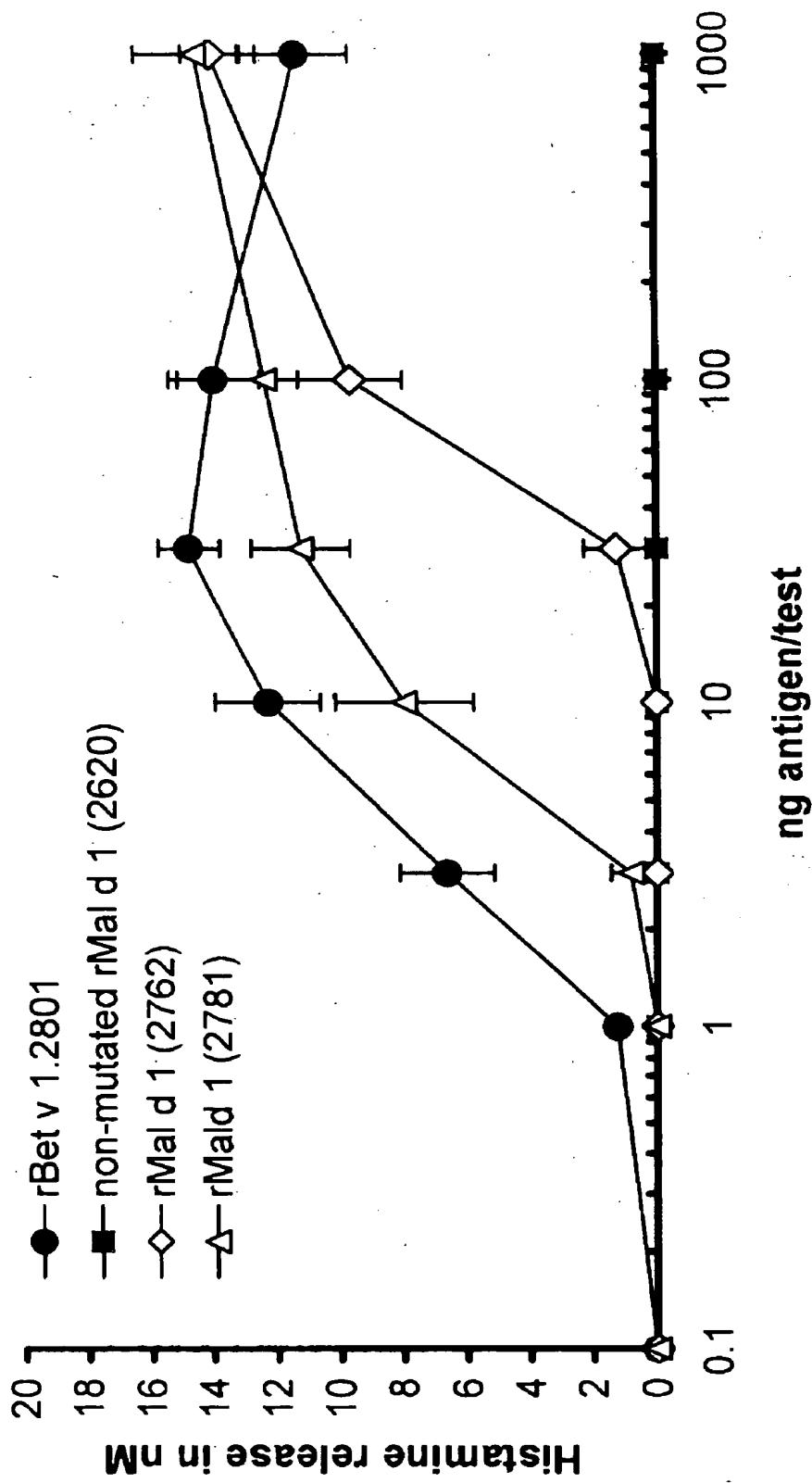


Figure 8B

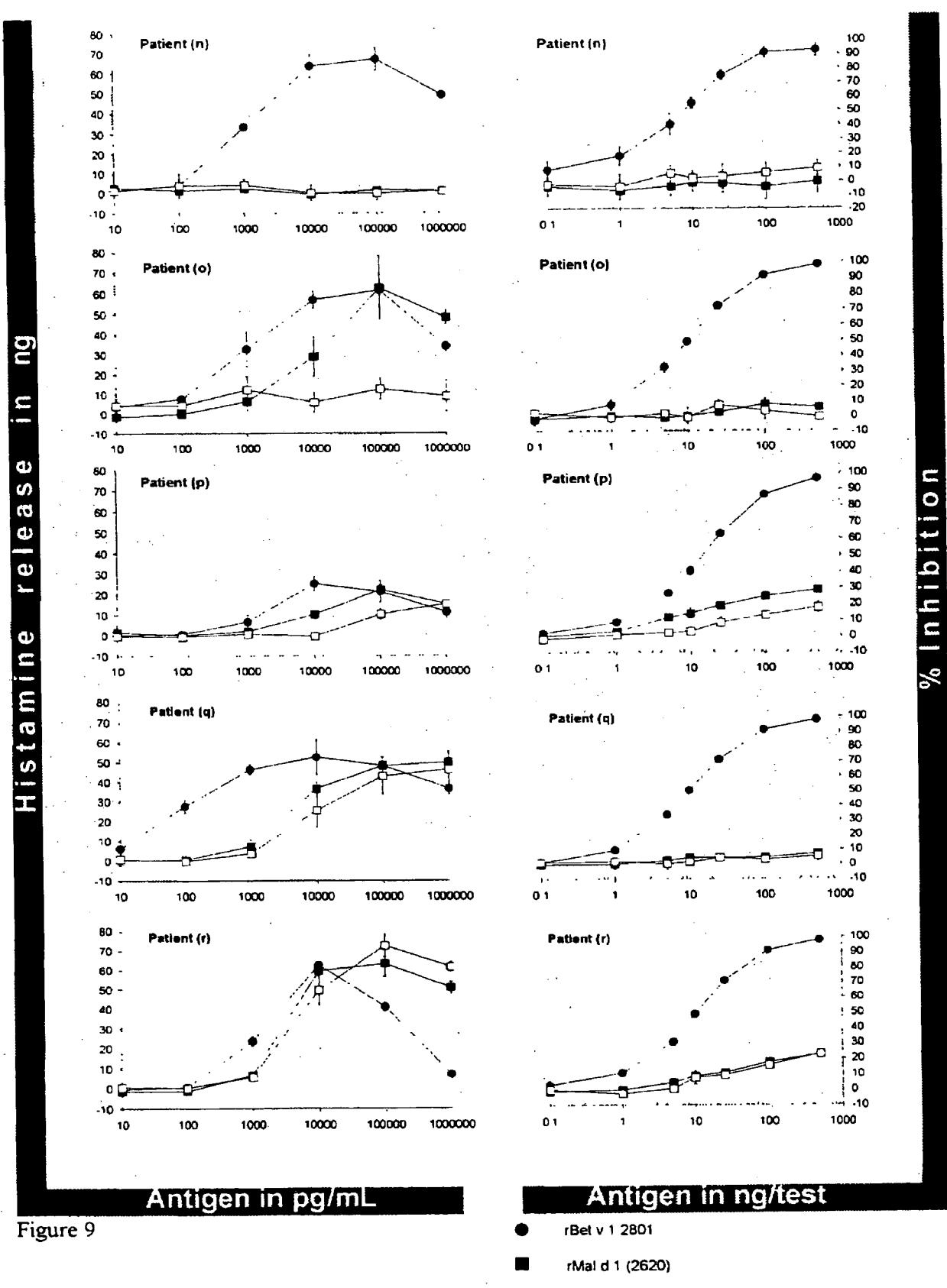
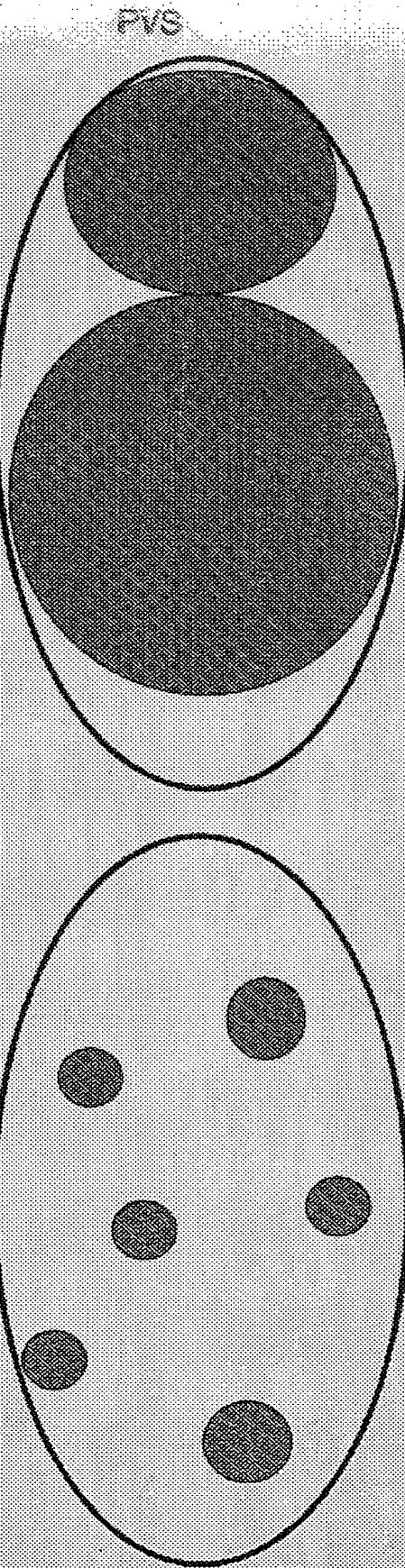


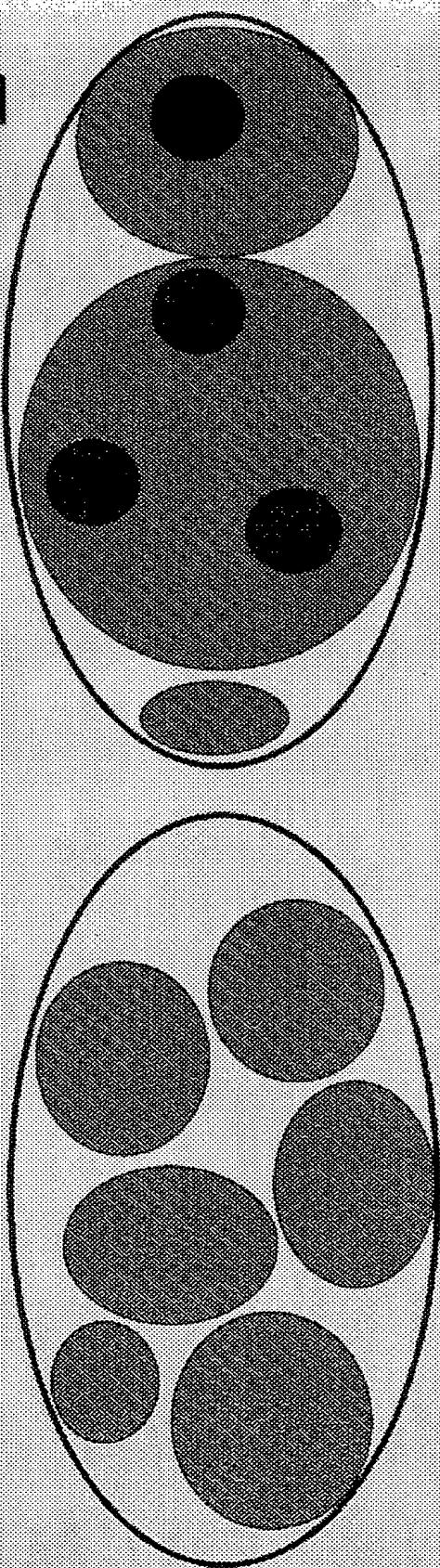
Figure 9

Scaffold protein: rMald 1 (2620) Front view

Scaffold protein: rMald 1 (2620) Back view



Derived mutant: Front view



Derived mutant: Back view

- Secondary mutations
- Bet v 1 specific surface structures
- Mald 1 specific surface structures

Figure 10A

Scaffold protein: rMald 1 (2620) Front view

Scaffold protein: rMald 1 (2620) Back view

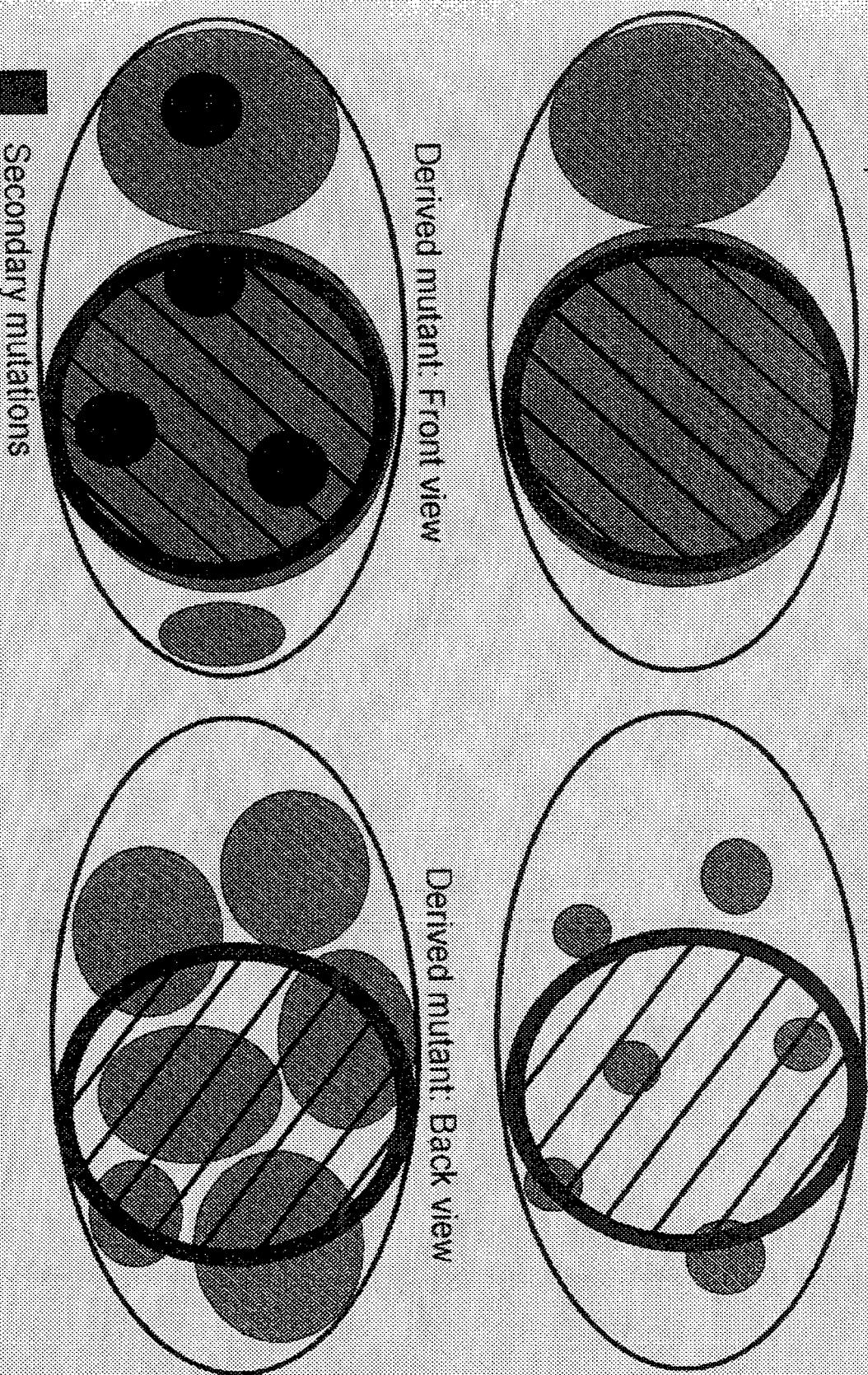


Figure 103

Modtaget

01 NOV. 2002

PVS

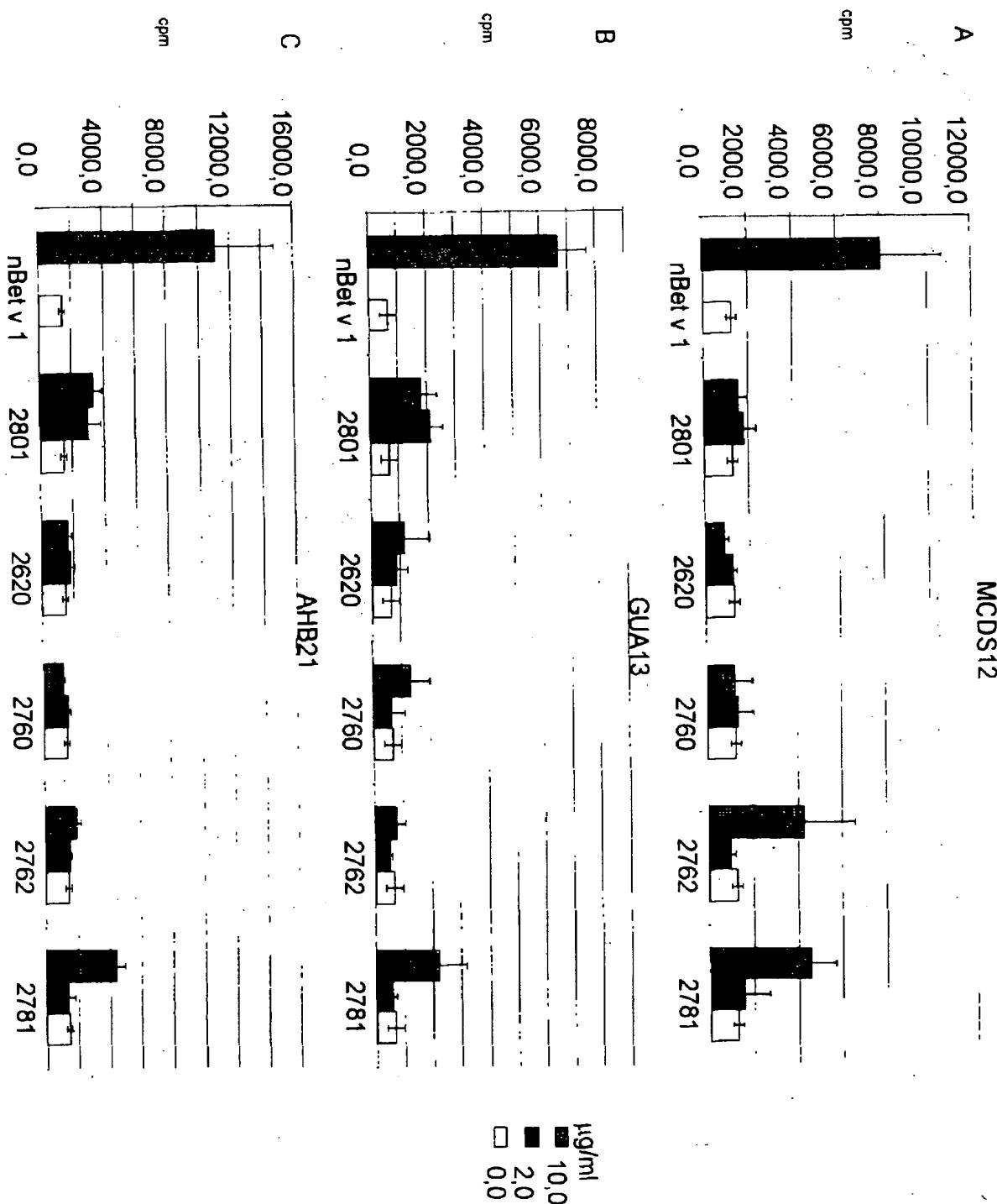


Figure 11

Modtaget

01 NOV. 2002

PVS

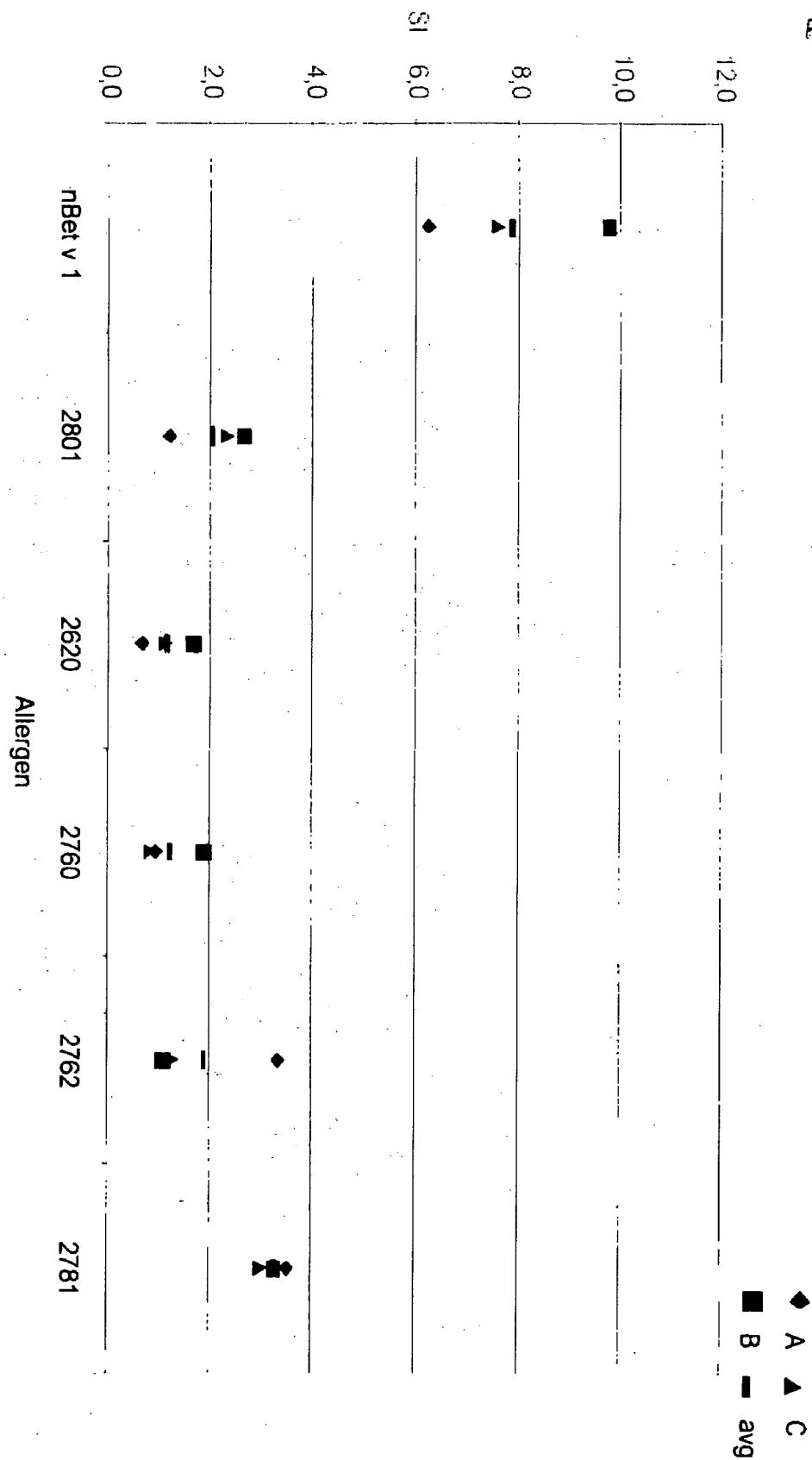


Figure 12

Modtaget

05 NOV. 2002

PVS

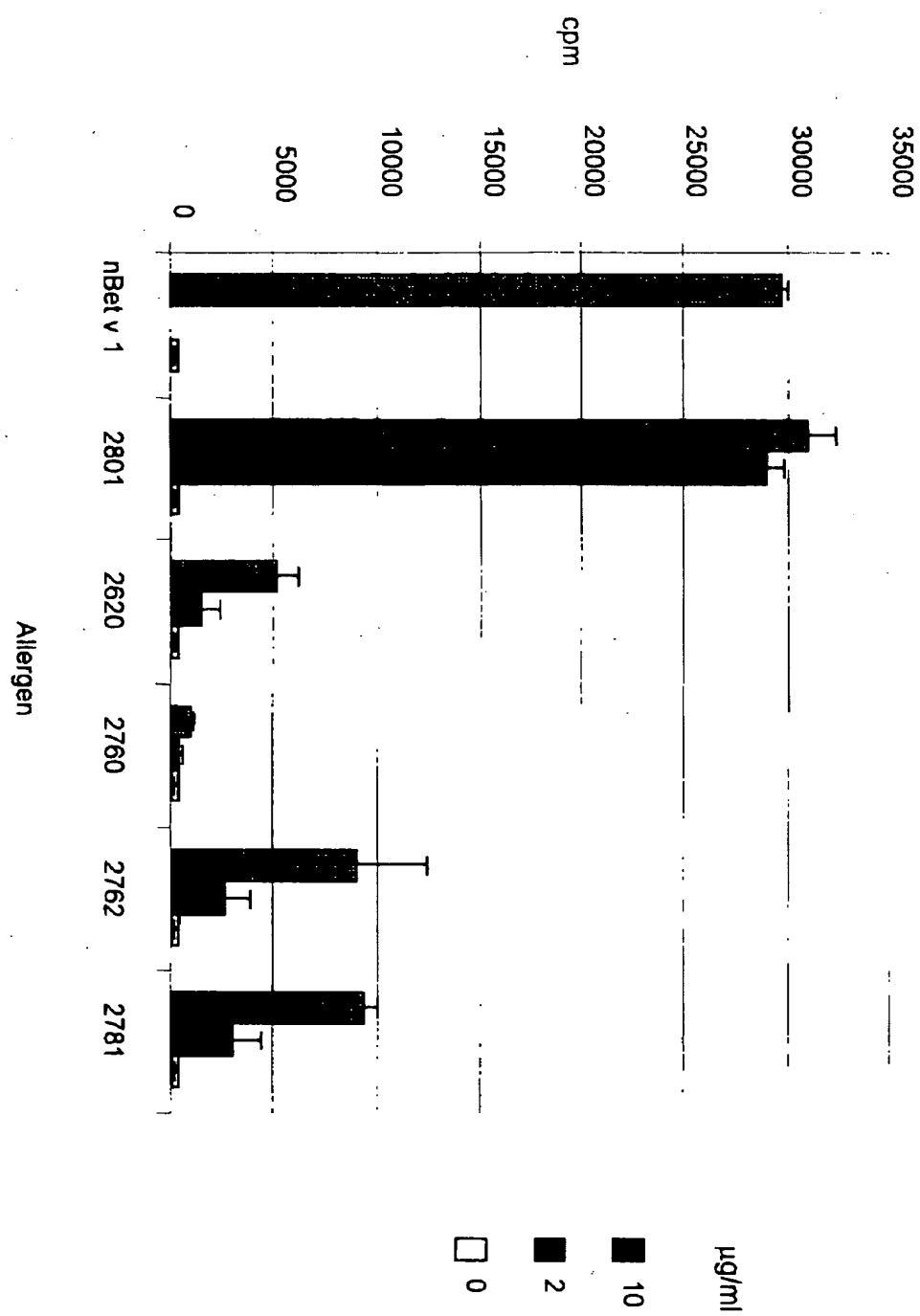


Figure 13

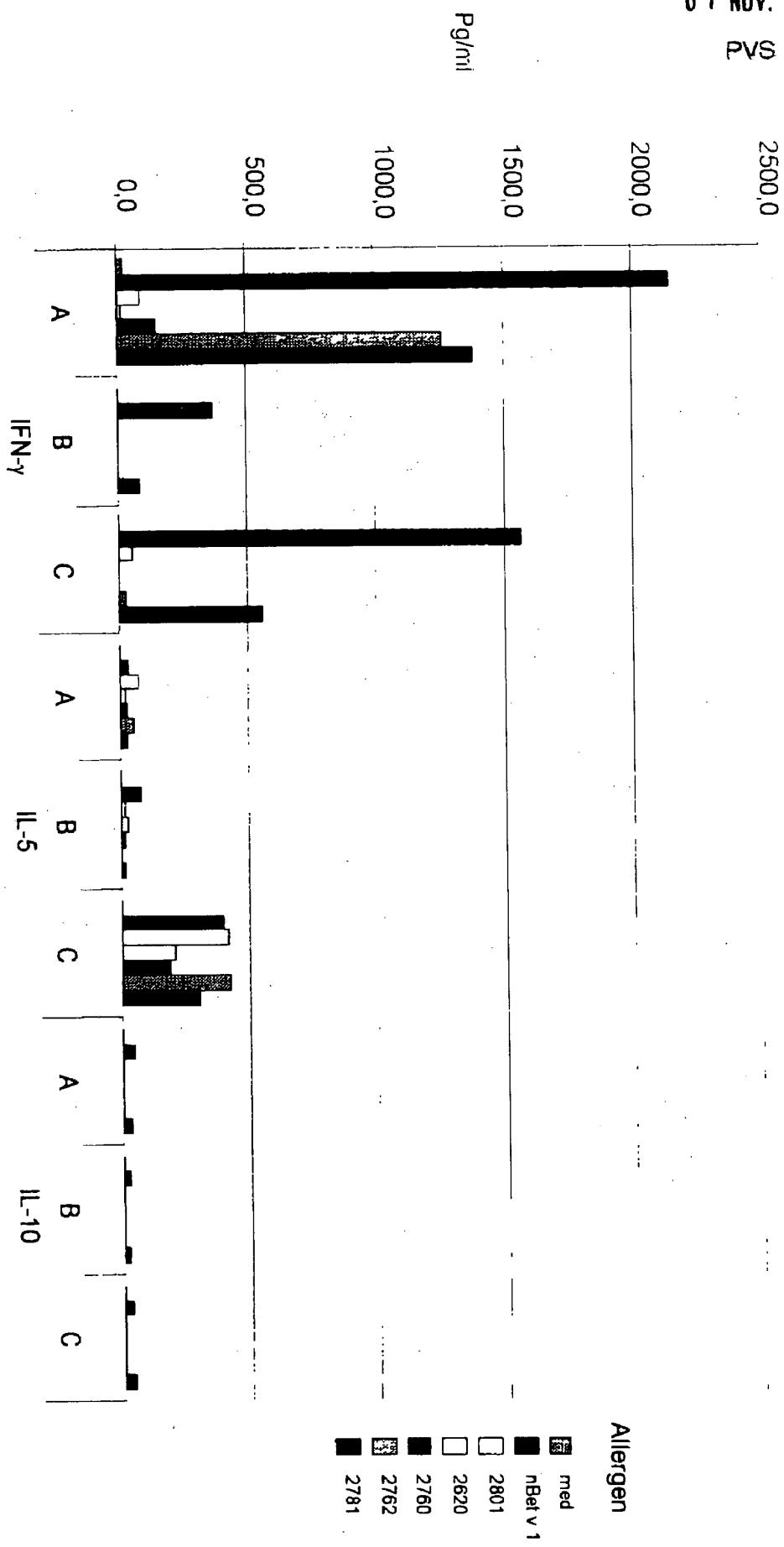


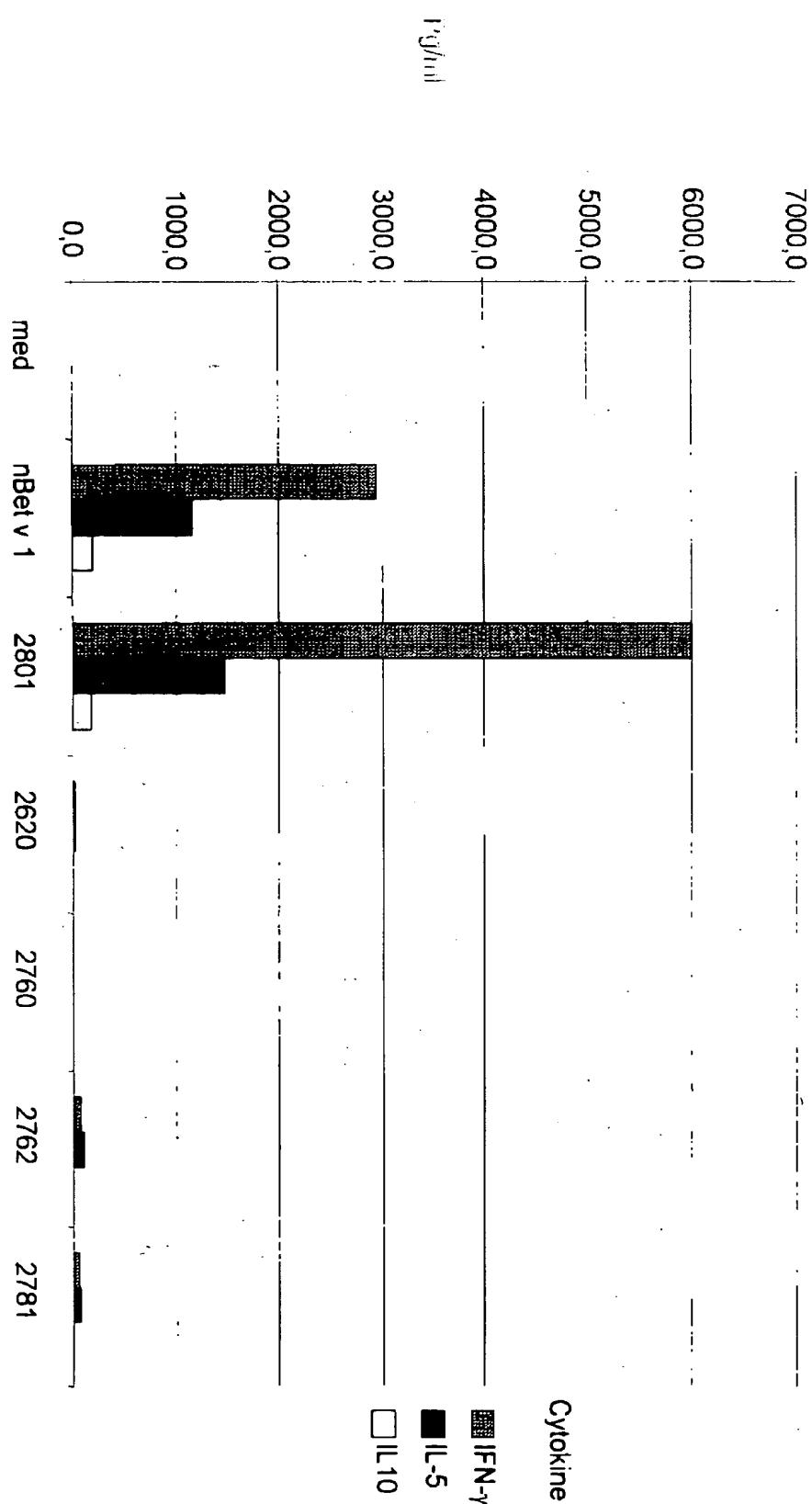
Figure 14

Modtaget

Modtaget

0 : NOV. 2002

PVS



Modtaget

NOV. 2002

PS

Pauc1 (T14325)
Betv1 2801 (280104)

1 0 2 0 3 0 4 0 5 0 6 0
G A Q S H S L E I T S S W S A E K I F S C I N L D V O T Y I P K A A T G A Y K S V E . M K G D G G A G T U R I I I L P E G
G V F N Y E T E T T I S V I P A A R L F K A F I L D G O N L F P K V A P Q A I S S V E N I E G N G G P G I K K I S F P E G
1 1 0 2 0 3 0 4 0 5 0 6 0

Pauc1 (T14325)

S P I T T M I V R T D A V N K E A L S Y D S T V I O G D I L G F I E S I E T H M V M V U P T A D G G S I T K T M A I P H I

Betv1 2801 (280104)

7 0 8 0 9 0 1 0 0 1 1 0 1 2 0
L P F K Y V K D R V D E V O H T N F K Y N Y S V I E G G P I G D T L E K I S N E I K I V A T P D G G S I L K I S N K Y H T
7 0 8 0 9 0 1 0 0 1 1 0 1 2 0

Pauc1 (T14325)

K G D A V V P E E N I K F A D A Q N T A L F K A I E A Y L I A M S D A Y N

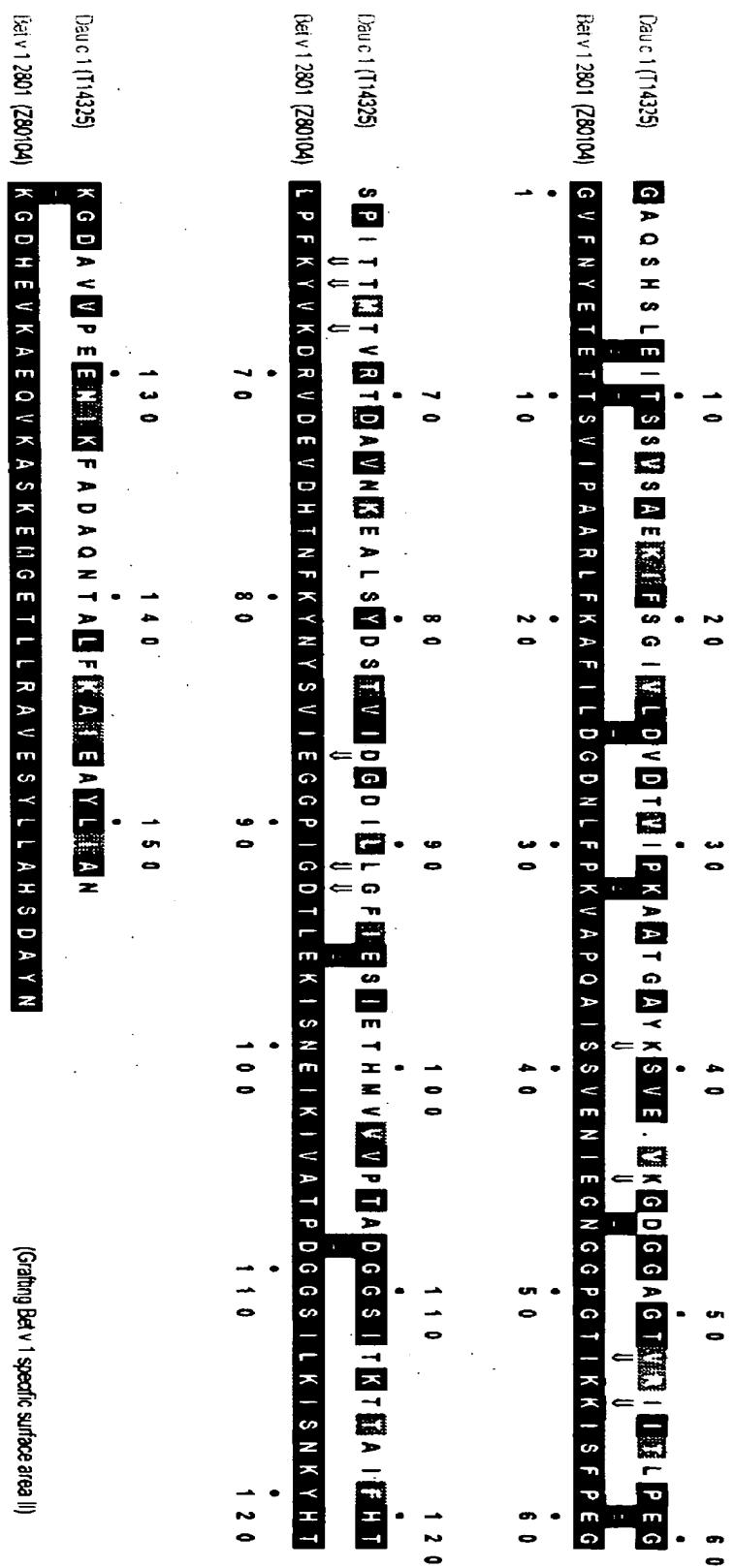
(Grafting Betv1 specific surface area)

Betv1 2801 (280104)

1 3 0 1 4 0 1 5 0
1 3 0 1 4 0 1 5 0

Figure 16

01 NOV. 2002



Modtaget

01 NOV. 2002

PVS

1 10 20 30 40 50 60
Lepd2(s66499) G K **N** T F **R** D C G H G E M E T E D D I S G C S G D T C V H P G Q K M T L B A K F A A N D T N K V T I K V L A K V A G T
Derp2(P49278) D Q V D V K D C A N H E I K P L P G C H G S E P C I I H R G K P F Q A L E A V F E A N Q H T K T A K I E I K A S I D G L
70 80 90 100 110 120
Lepd2(s66499) T **N** O V P G C E T D G C K V L K C P R K K G E A L D F N Y G M I I P A T I P E I K - A D V T A E L V G D H G V M A C G T I H G O - V E
Derp2(P49278) E **N** D V P G I D P H A C H Y H C P L Y K G Q D I K Y T W H V P K I A P K S E H V V T E K V M G D D G V L A C A I A T H A K I R D
70 80 90 100 110 120

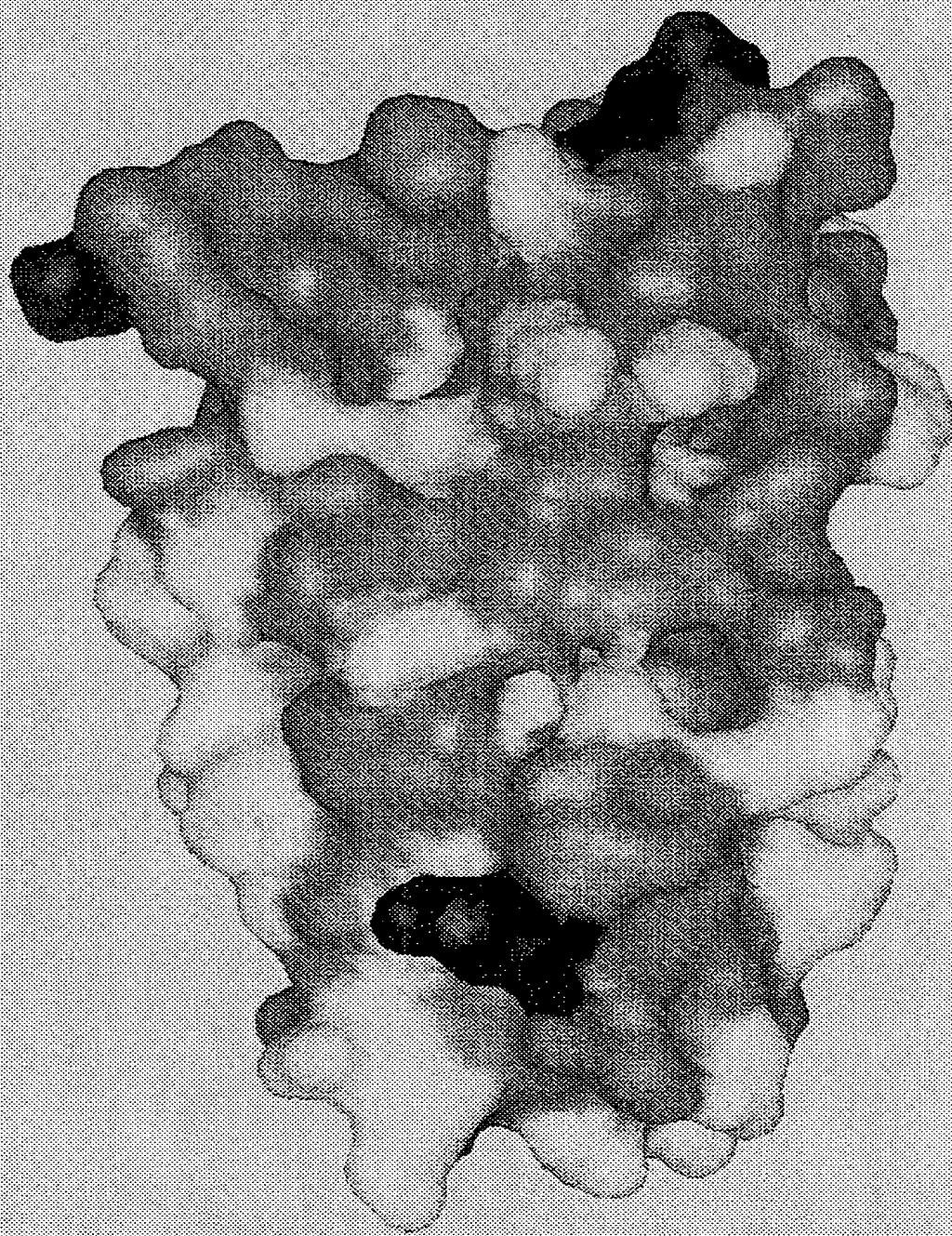
Figure 18

Modtaget

01 NOV. 2002

PVS

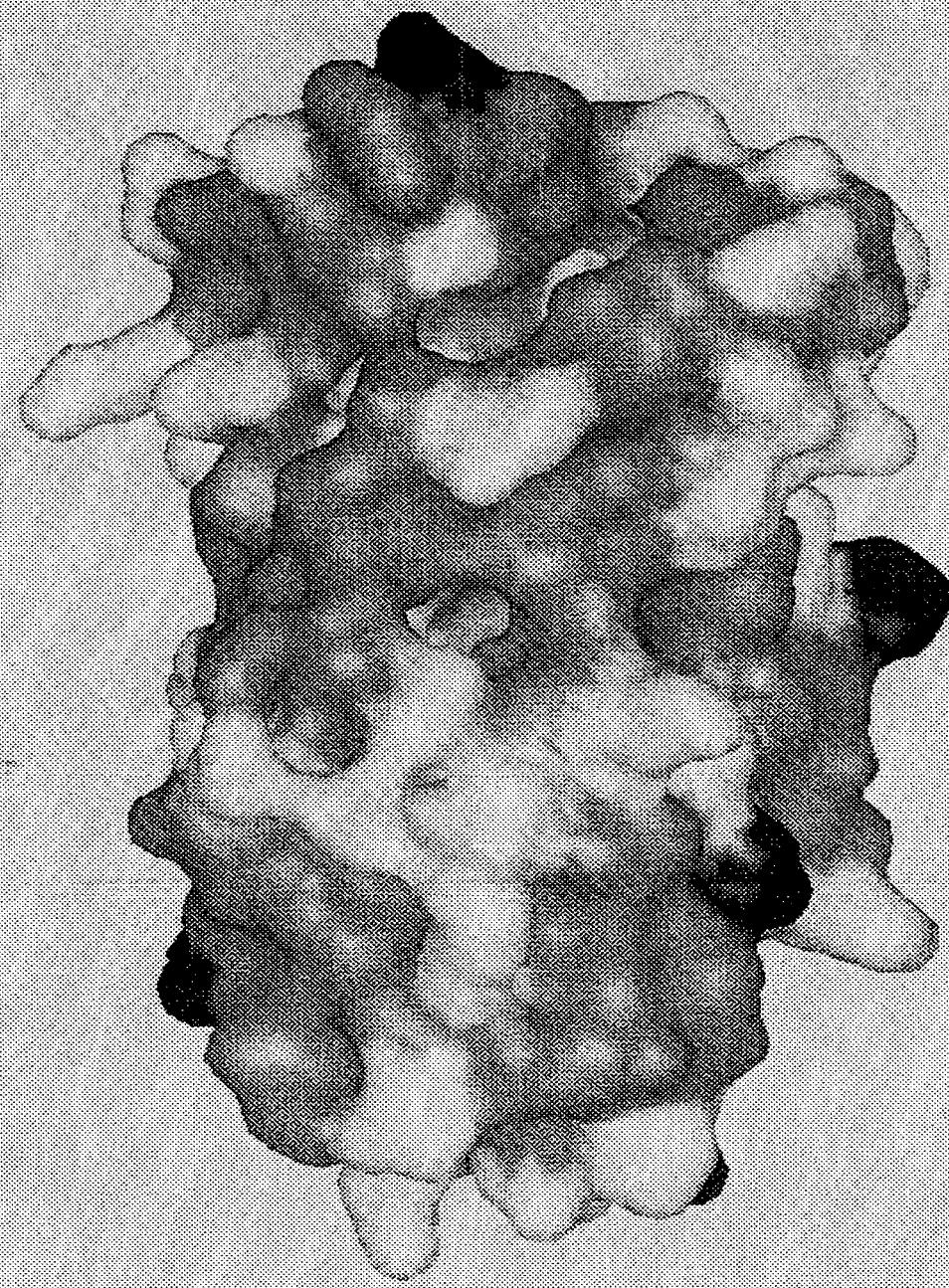
Figure 3a from (6)



Modtaget

01 NOV. 2002

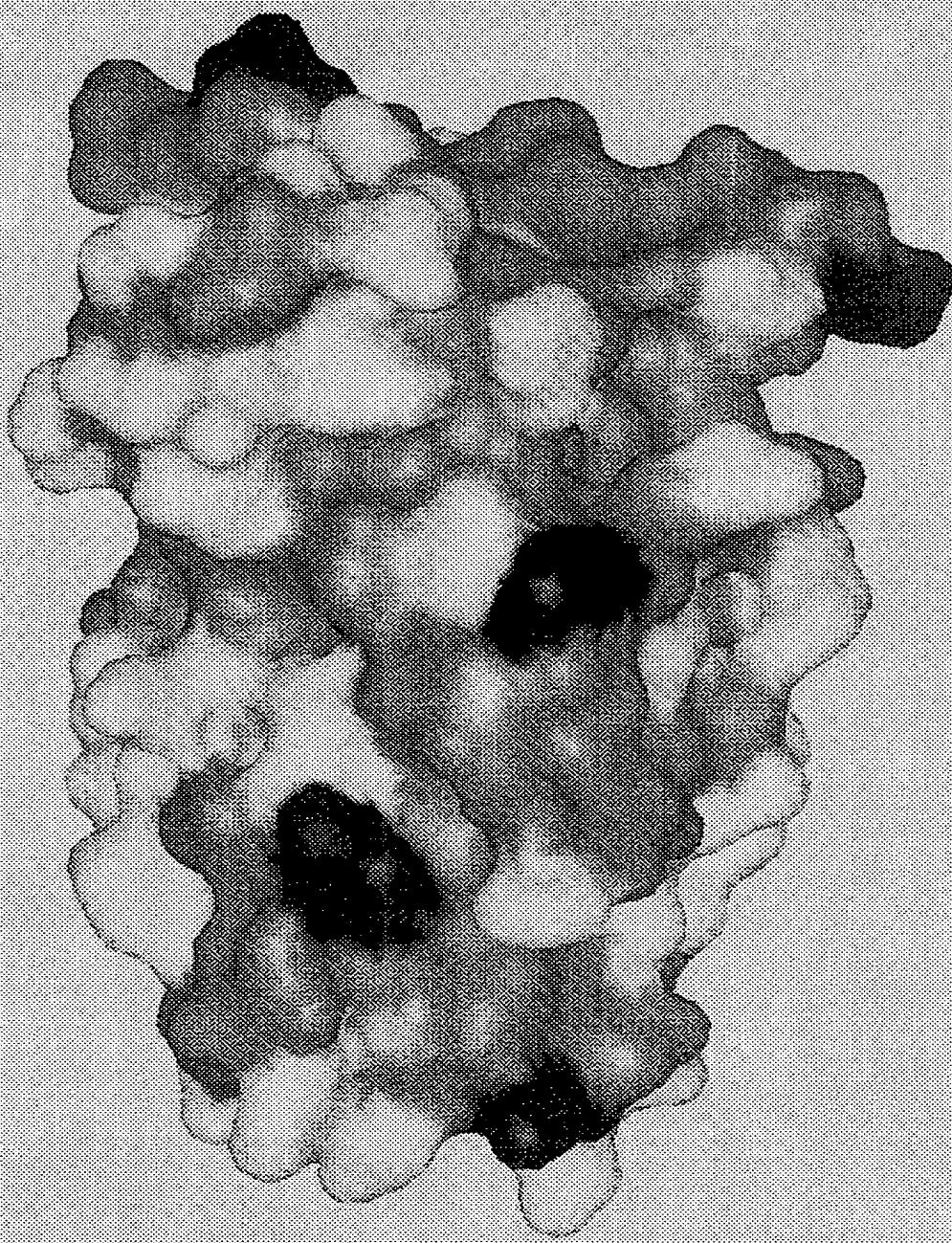
Skane 1987um 1 (90)



Medtaget

01 NOV. 2002

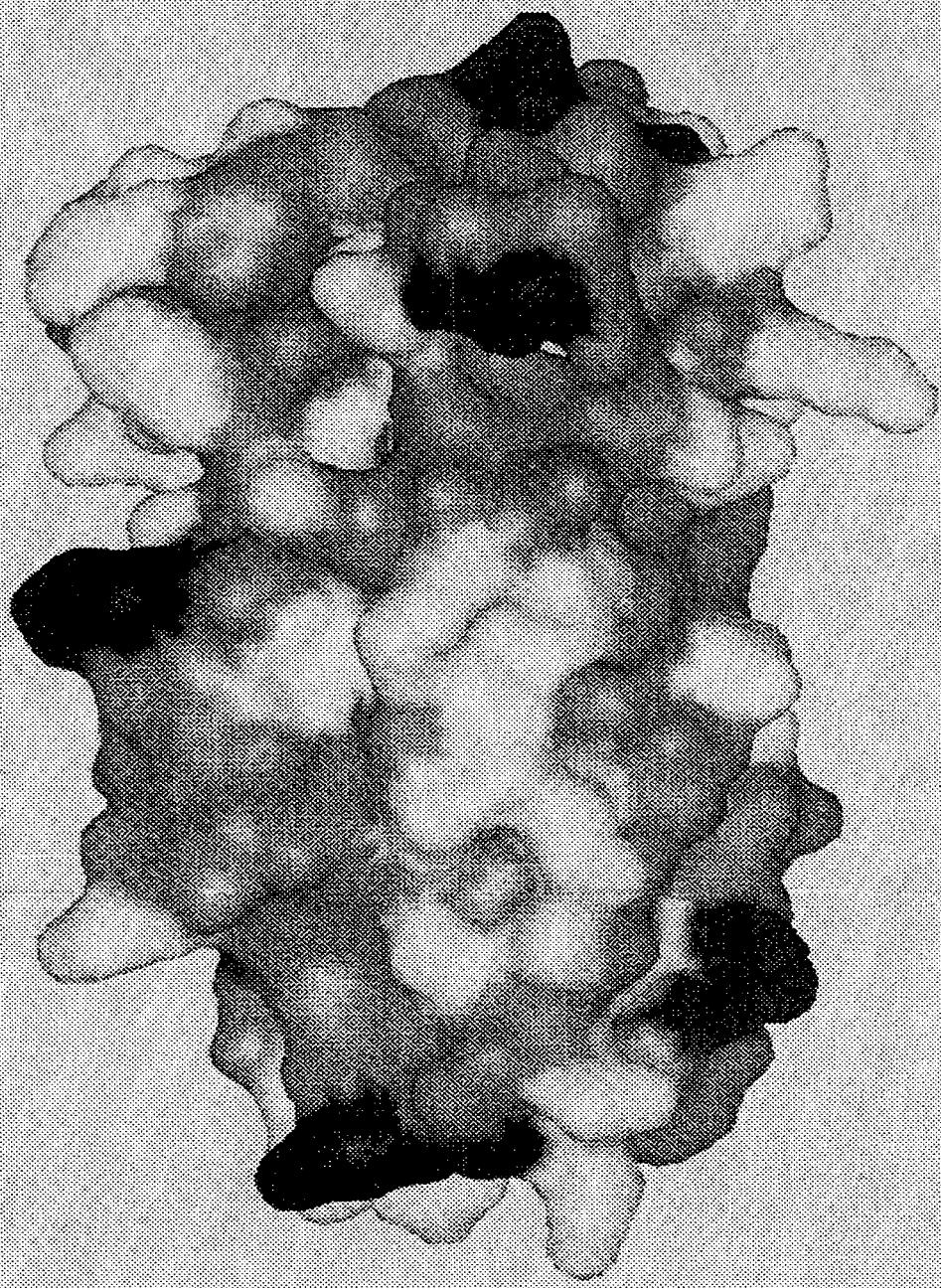
192 11112 2 (180°)



Modtaget

01 NOV. 2002

pre



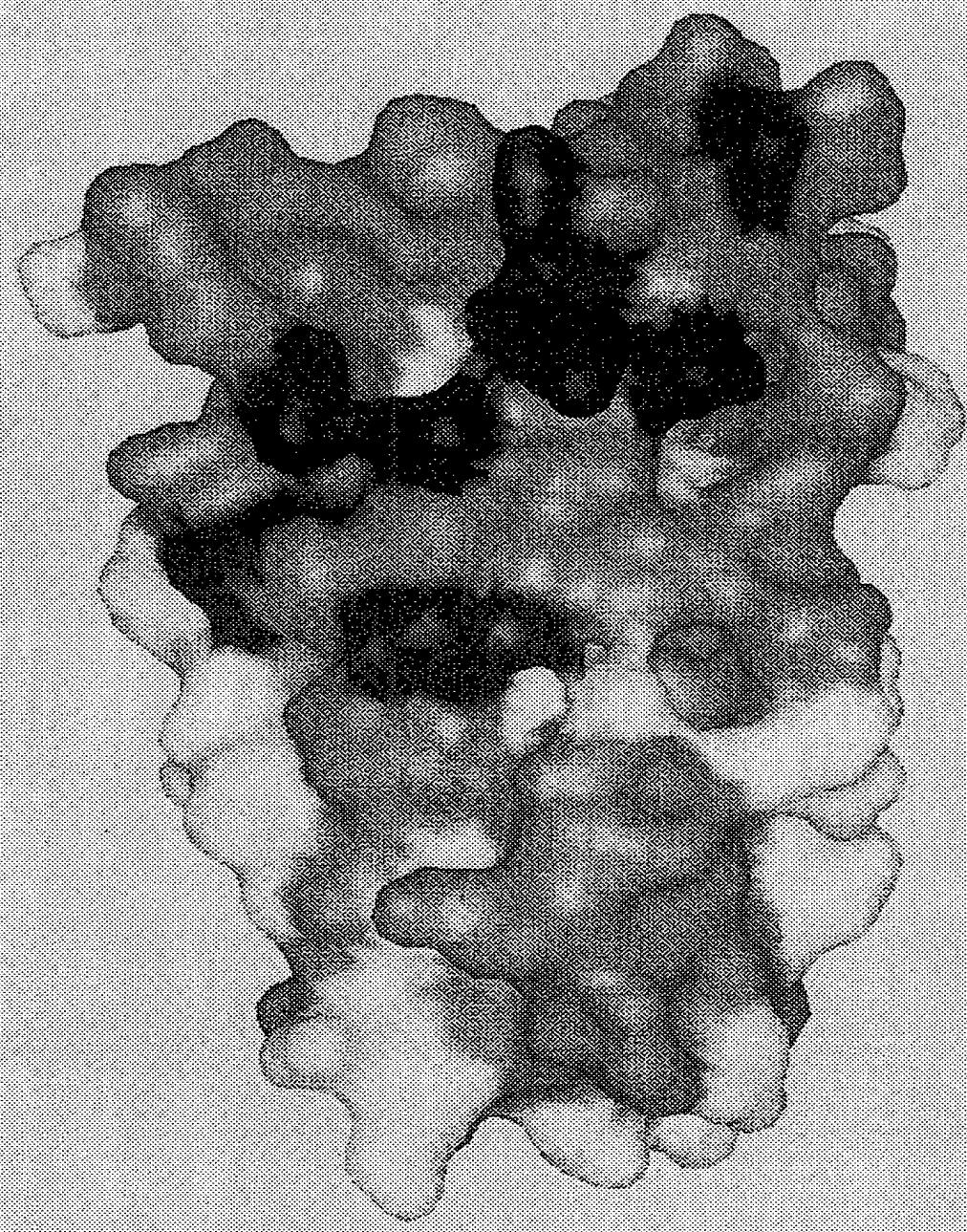
(s0L2) 2 UNITS Q61 serial #

Modtaget

01 NOV. 2002

PMS

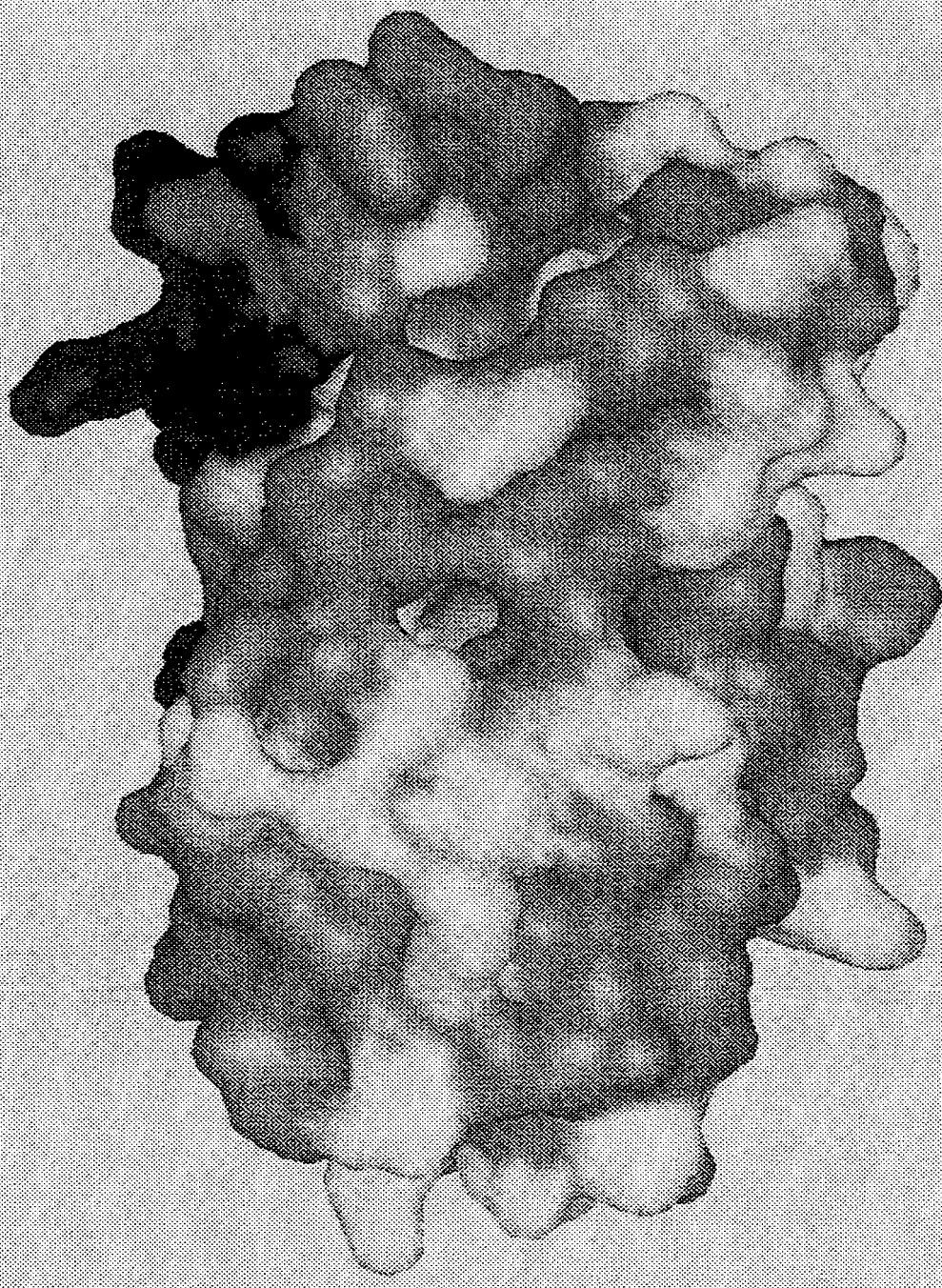
DK 0002 3702 0000 0000



Modtaget

01 NOV. 2002

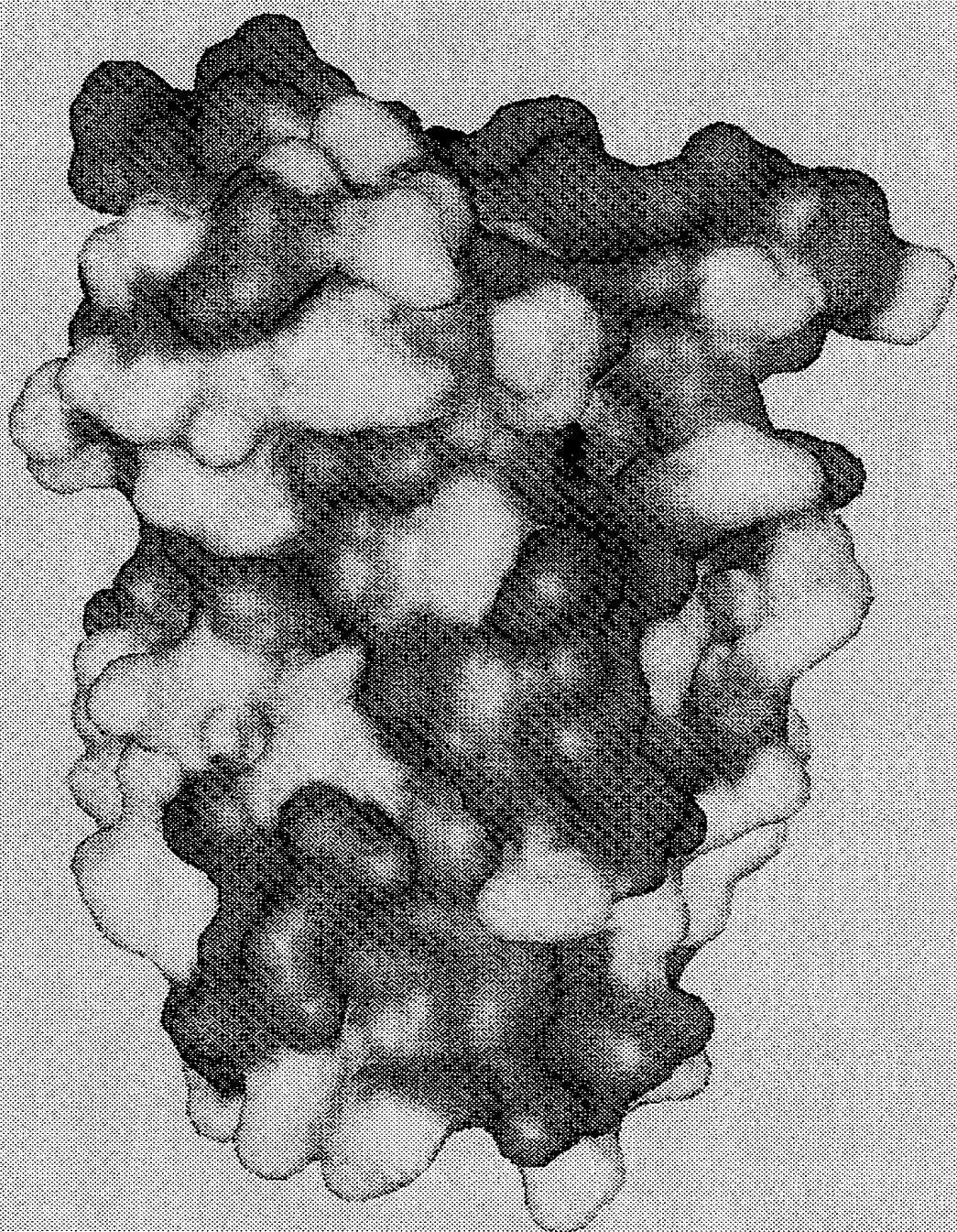
ENIQUE 2002 Crystallizing Turn 1 (90°)



Modtaget

9 NOV. 2002

PVC

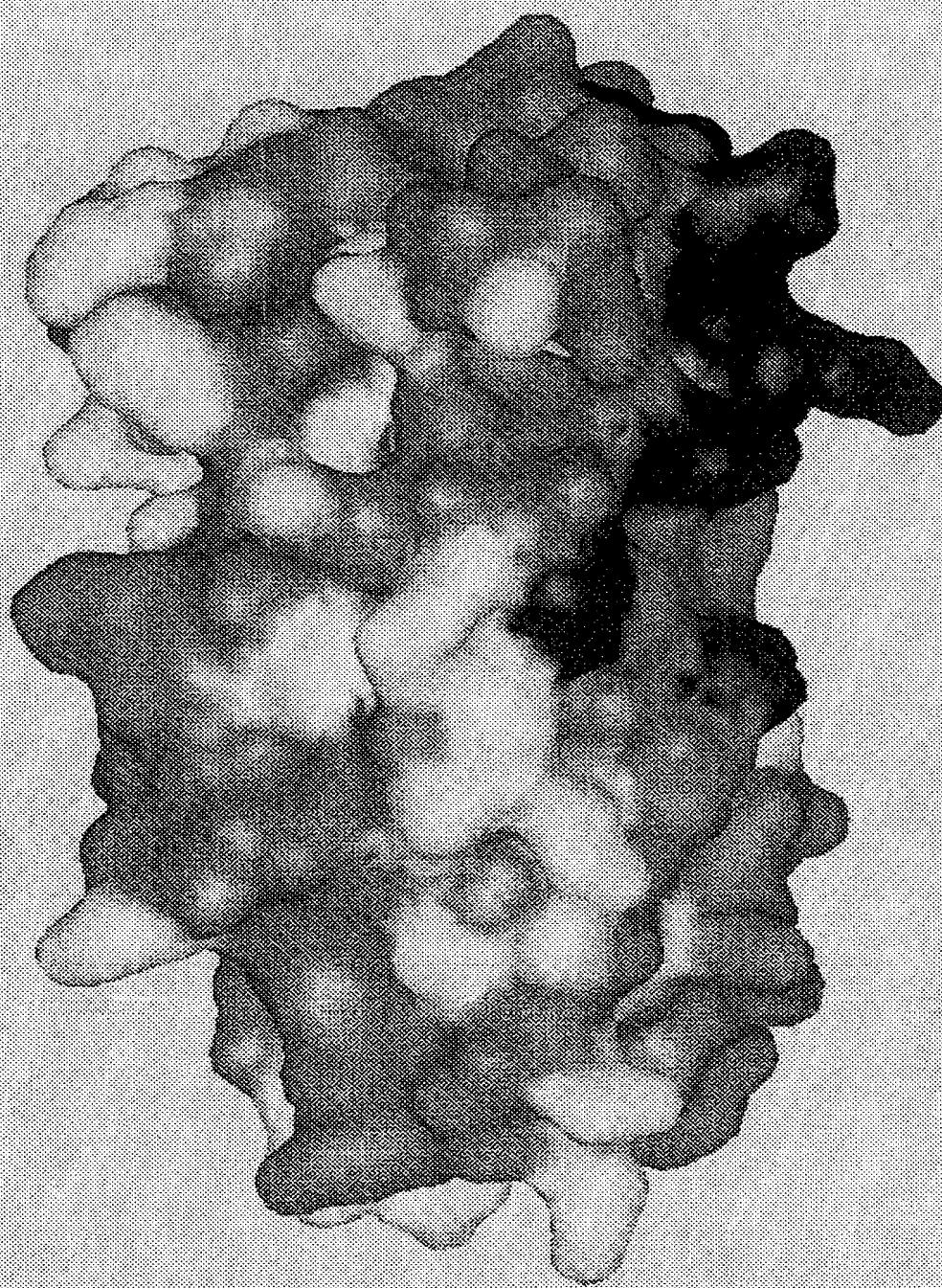


081 (1981) 2002 Correction Turn 2

01 NOV. 2002

PIC

FIGURE 200 Grafting Turn 3 (270°)



Modtaget

01 NOV. 2002

PVS

Meld 1 (2620) (A488060)

G V Y T Y E N E Y T S E I P P P R L F K A F V L D A D N L I P K I A P Q A I K H A E I L E G G D G G P G T I K K I T F G E

Bet v1.2801 (280104)

1	10	20	30	40	50	60
1	10	20	30	40	50	60

Meld 1 (2620) (A488060)

G S Q Y G Y V K H K I D S V D E A N Y S Y A Y I L I E G D A L T O T I E K V S V E T K L V A S . G S G S I I K S I S H Y

Bet v1.2801 (280104)

7.0	8.0	9.0	10.0	11.0
7.0	8.0	9.0	10.0	11.0

Meld 1 (2620) (A488060)

H T K G D V E T M E E H V K A G K E K A H G L F K L I E S Y L K D H P D A Y N .

Bet v1.2801 (280104)

12.0	13.0	14.0	15.0
12.0	13.0	14.0	15.0

Figure 21

Modtaget

D 1 NOV. 2002

PVS

Glyd2(A4927216) G **W** K **F** K D C G K G E V T E L D I T D C S G - **W** F C W I H R G K P L I L E A K F A A N Q D I T K A T I K **W** L A K Y A G
Dsp2(P49278) D Q V D V K D C A N H E I K K V L V P G C H G S E P C I I H R G K P F Q L E A V F E A N Q T K T A K I E - K A S I D G
1 10 20 30 40 50
6 0 7 0 8 0 9 0 10 0 11 0 12 0
7 0 8 0 9 0 10 0 11 0 12 0

Figure 22

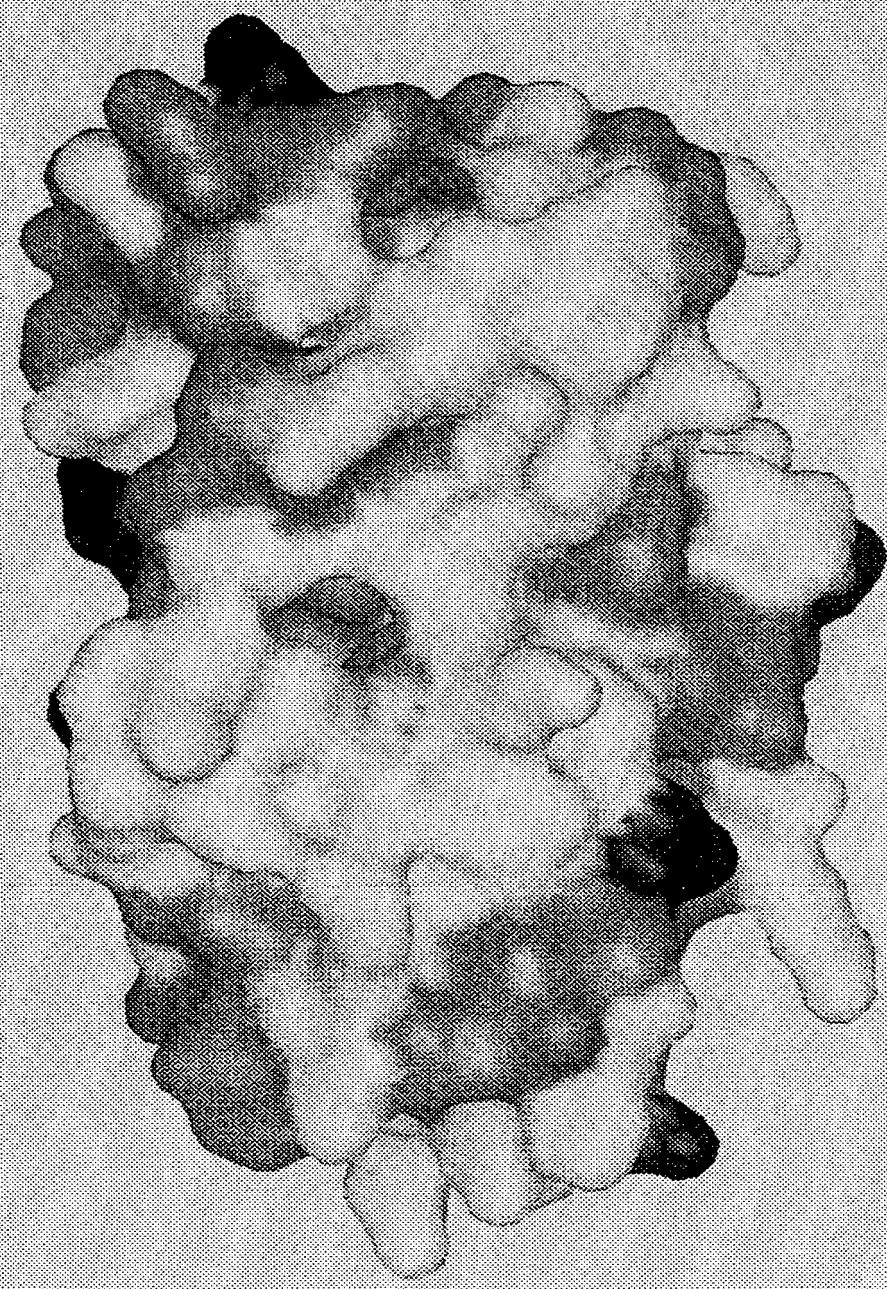
Figure 23 A front (0°)



8-1-2002

PS

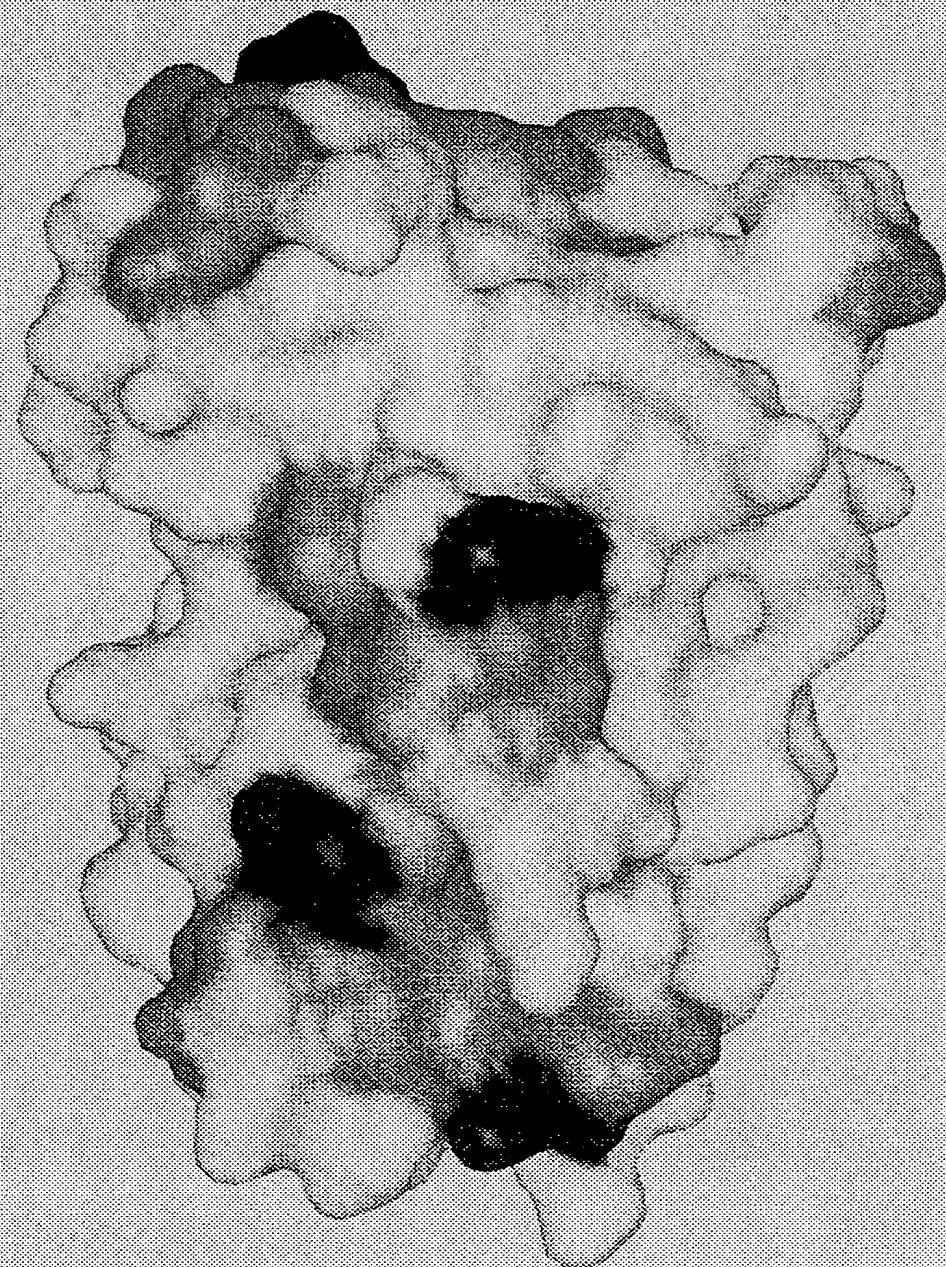
Figure 24B (Part 1 (9))



01.09.2002

25

Figure 23C Tum 2 (180°)

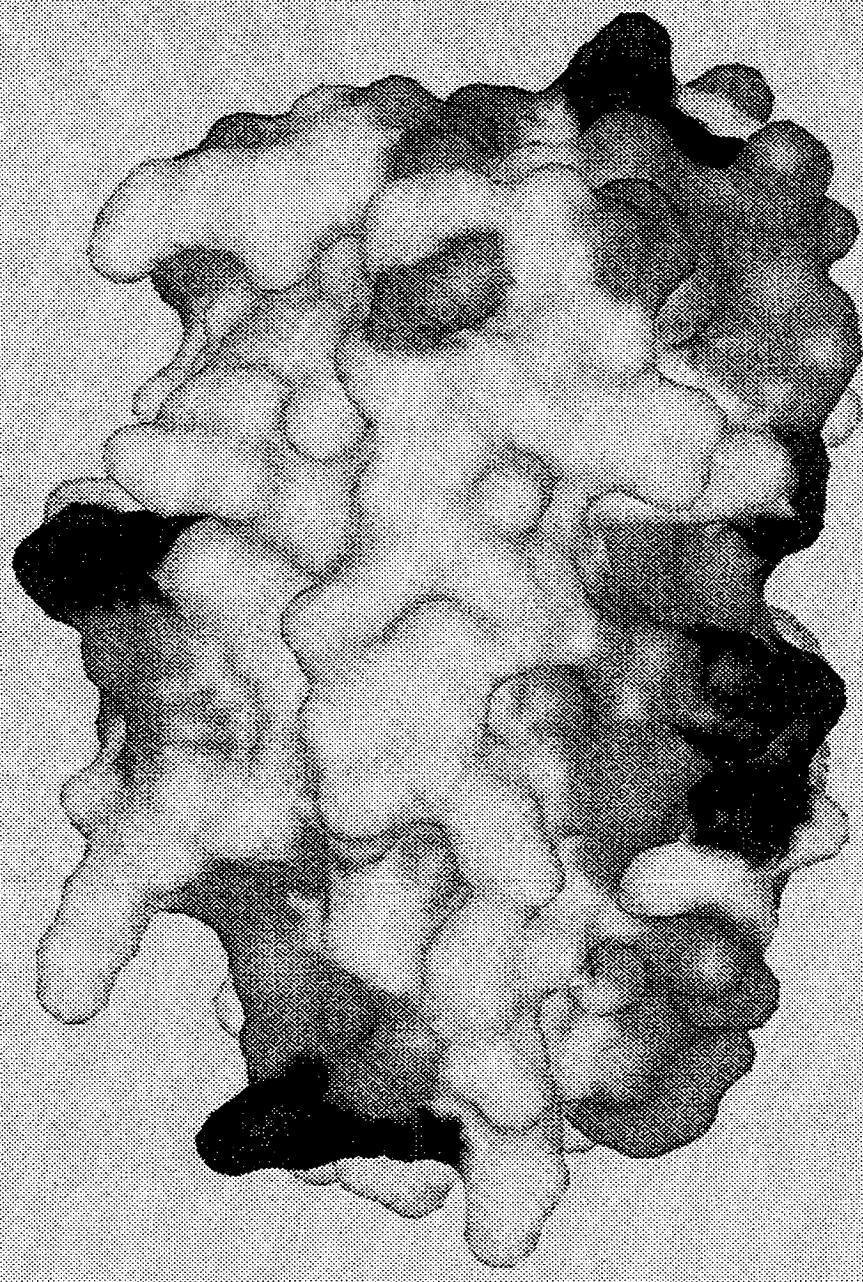


Modtaget

8 1 NOV. 2002

PVS

Figure 23D Turn 3 (230⁰)



248

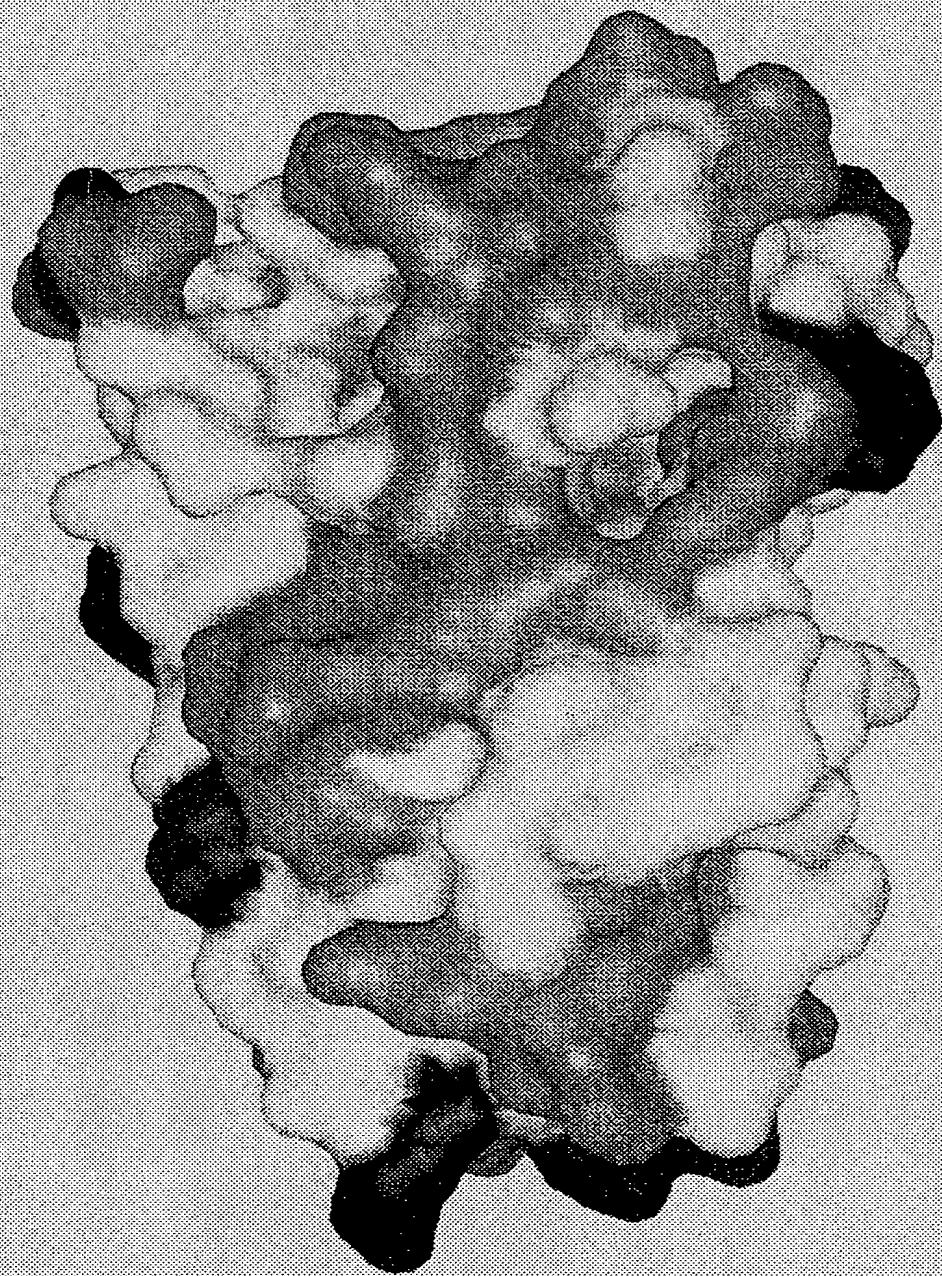
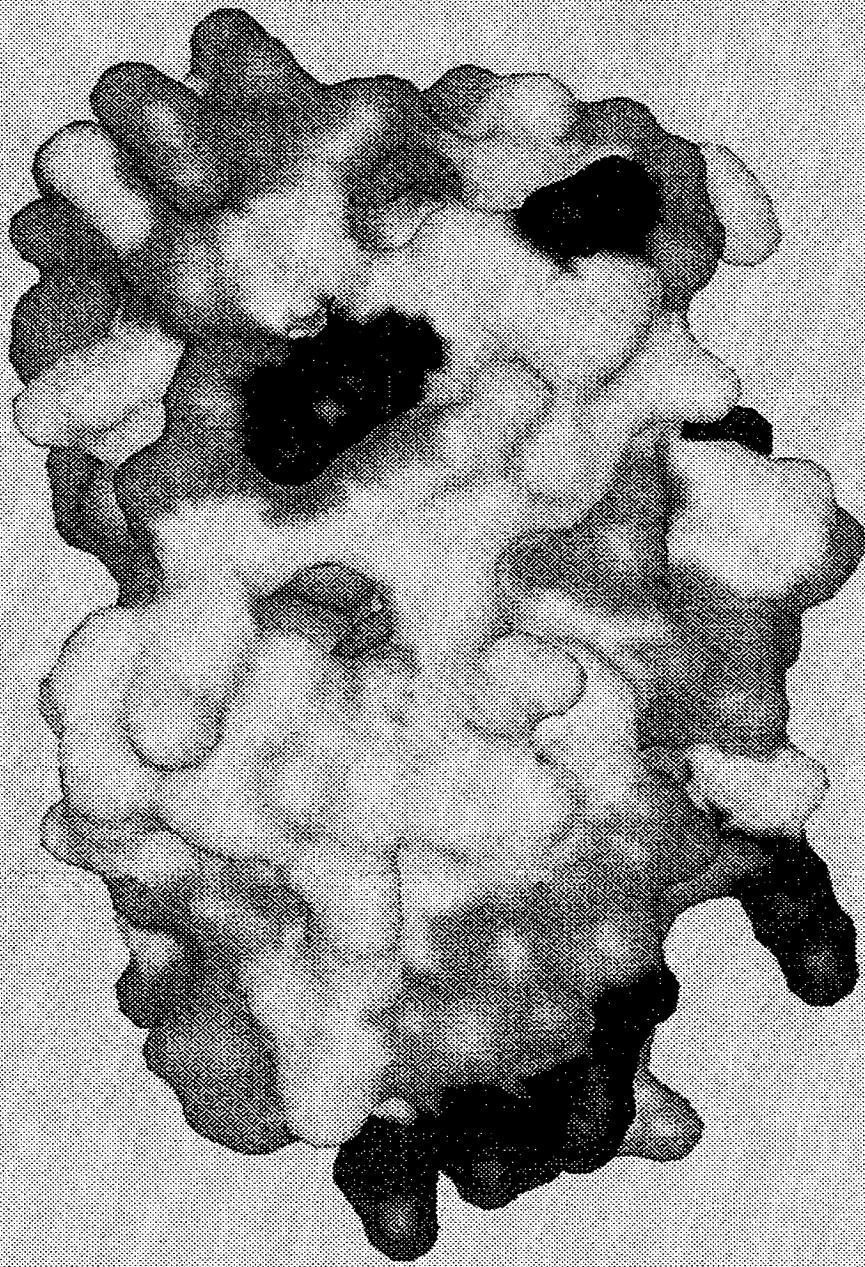


Figure 24 A grafting foam (0°)

01 NOV. 2012

FIG

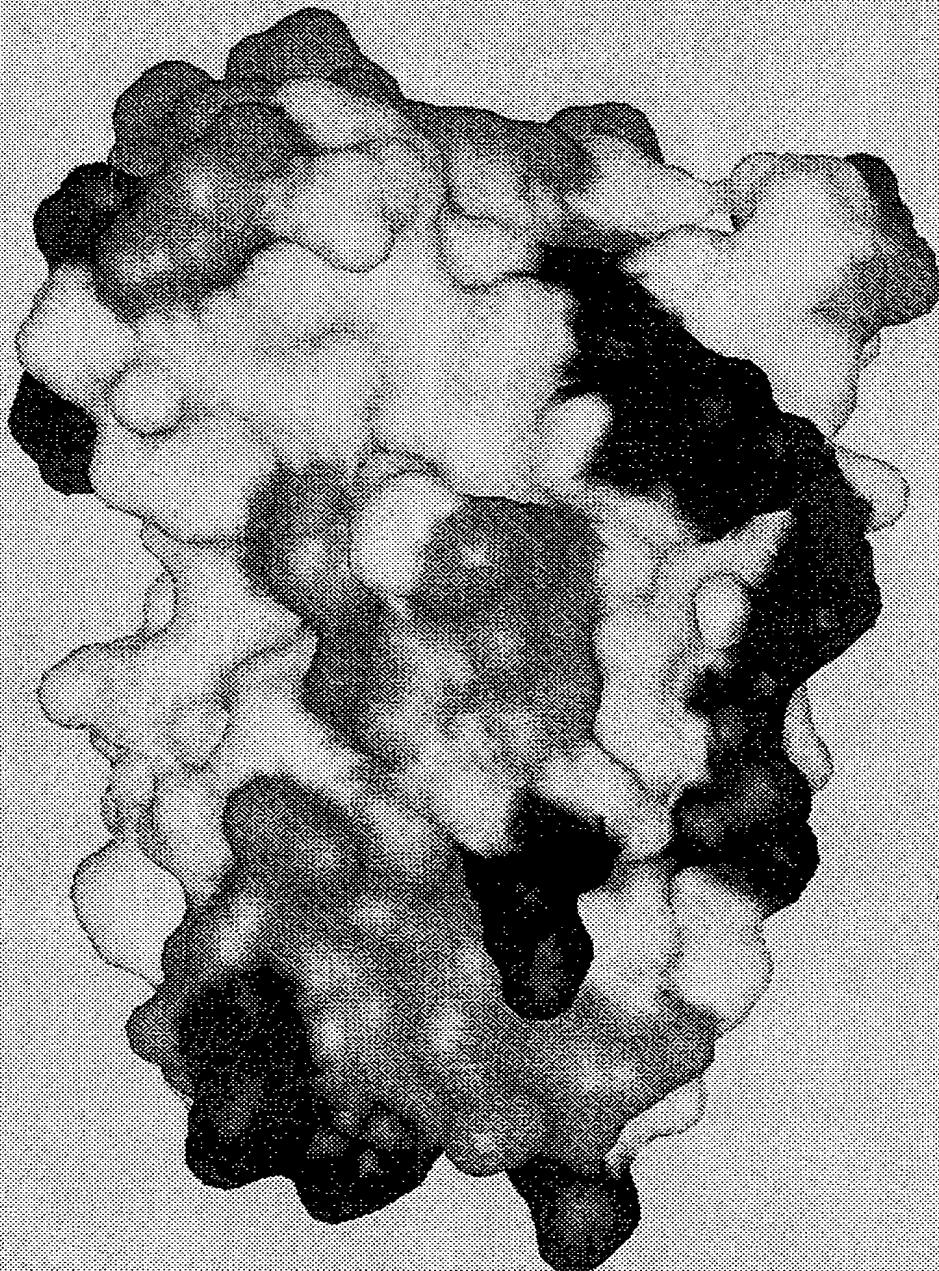
Figure 24B scanning Part 1 (90°)



01 NOV. 2002

ANS

Figure 24C, Rating, Turn 2 (180°)



Modtaget

8.11.2002

2002

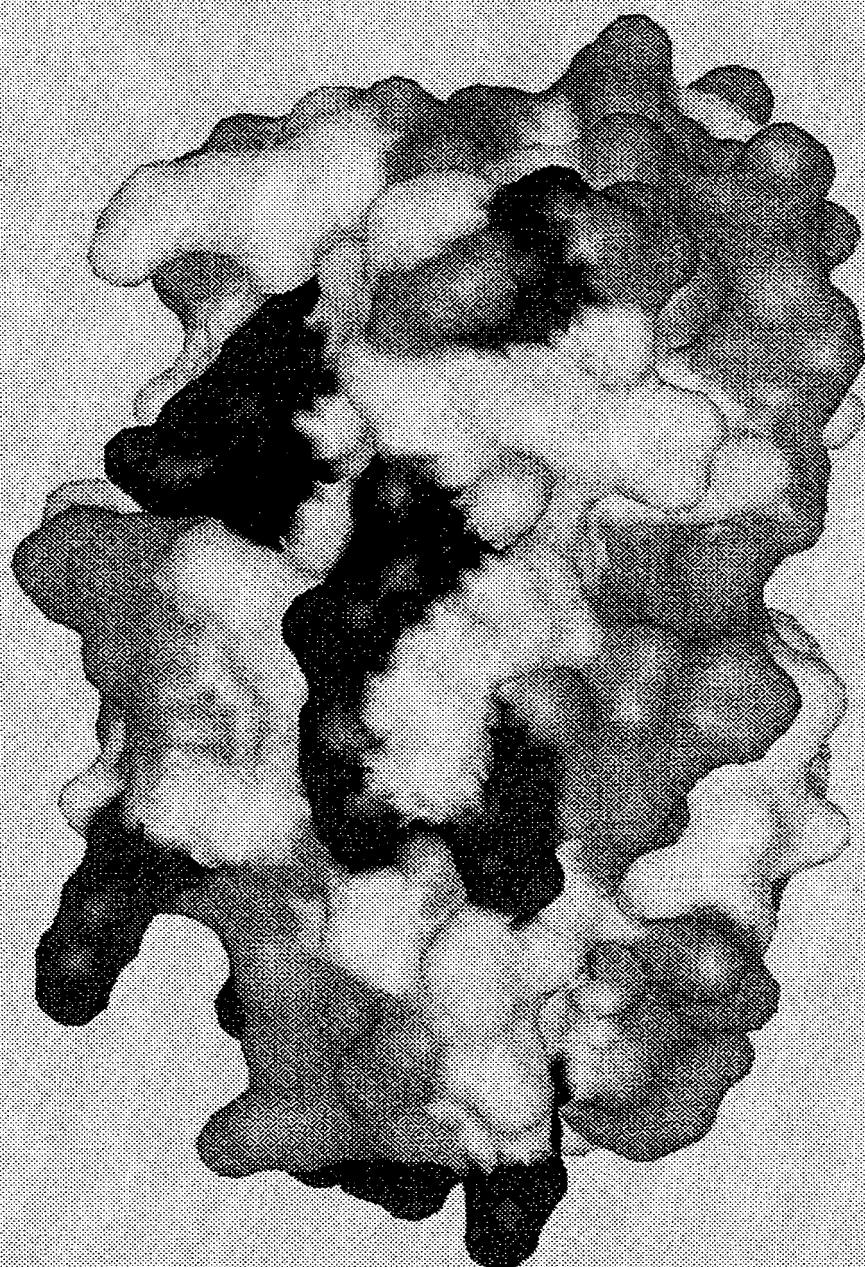


FIGURE 24D rendering, Turn 3 (270°)

Comparison of secondary structures (CD spectra deconvolutions)

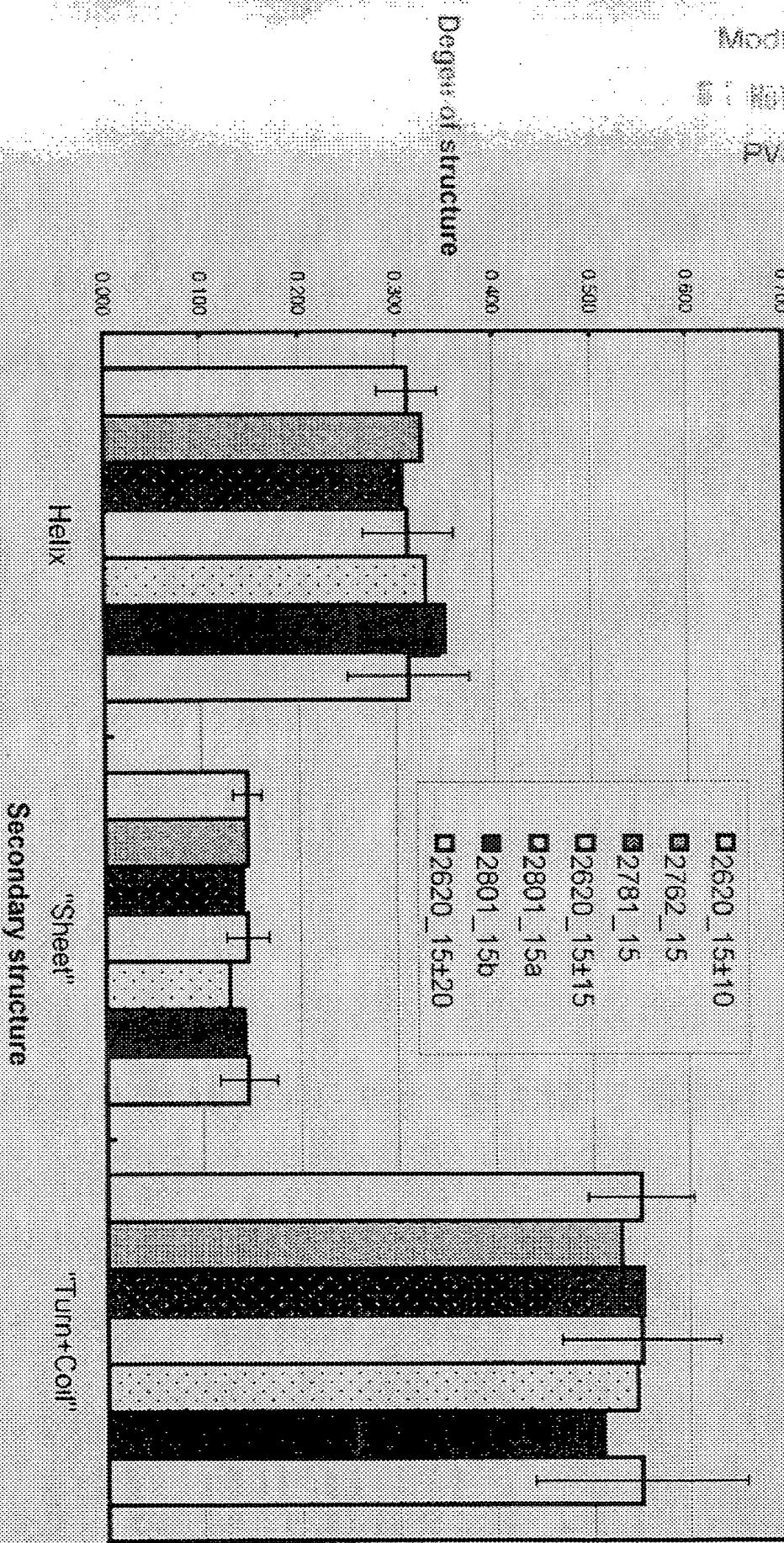


Figure 25

Modtaget

01 NOV. 2002

PVS

P200101752 sekvensliste ST25
SEQUENCE LISTING

<110> ALK-ABELLÓ A/S

<120> Recombinant protein variants

<130> P200101752 DK

<160> 3

<170> PatentIn version 3.1

<210> 1

<211> 158

<212> PRT

<213> Malus domestica

<400> 1

Gly Val Tyr Thr Tyr Glu Asn Glu Tyr Thr Ser Glu Ile Pro Pro Pro
1 5 10 15

Arg Leu Phe Lys Ala Phe Val Leu Asp Ala Asp Thr Leu Ile Pro Gln
20 25 30

Ile Ala Pro Gln Ala Ile Lys His Ala Glu Ile Leu Ser Gly Asp Gly
35 40 45

Gly Pro Gly Thr Ile Lys Lys Ile Thr Phe Gly Glu Gly Ser Gln Tyr
50 55 60

Gly Tyr Val Lys His Lys Ile Asp Ser Val Asp Glu Ala Asn Tyr Ser
65 70 75 80

Tyr Ala Tyr Thr Leu Ile Glu Gly Asp Ala Leu Thr Asp Thr Ile Glu
85 90 95

Lys Val Ser Tyr Glu Thr Lys Leu Val Ala Ser Gly Ser Gly Ser Ile
100 105 110

Ile Lys Ser Ile Ser His Tyr His Thr Lys Gly Asp Val Glu Ile Met
115 120 125

Glu Glu His Val Lys Ala Gly Lys Glu Lys Ala His Gly Leu Phe Lys
130 135 140

Leu Ile Glu Ser Tyr Leu Lys Asp His Pro Asp Ala Tyr Asn
145 150 155

<210> 2
<211> 158
<212> PRT
<213> Malus domestica

<400> 2

Gly Val Tyr Thr Tyr Glu Asn Glu Tyr Thr Ser Glu Ile Pro Pro Pro
1 5 10 15

Arg Leu Phe Lys Ala Phe Val Leu Asp Ala Asp Asn Leu Ile Pro Lys
20 25 30

Ile Ala Pro Gln Ala Ile Lys His Ala Glu Asn Ile Glu Gly Asn Gly
35 40 45

Gly Pro Gly Thr Ile Lys Lys Ile Thr Phe Gly Glu Gly Ser Gln Tyr
50 55 60

Lys Tyr Val Lys His Arg Ile Asp Ser Val Asp His Ala Asn Tyr Ser
65 70 75 80

Tyr Ala Tyr Thr Leu Ile Glu Gly Asp Ala Leu Thr Asp Thr Ile Glu
85 90 95

Lys Val Ser Tyr Glu Thr Lys Leu Val Ala Ser Gly Ser Gly Ser Ile
100 105 110

Ile Lys Ser Ile Ser His Tyr His Thr Lys Gly Asp Val Glu Ile Met
115 120 125

Glu Glu His Val Lys Ala Gly Lys Glu Lys Ala His Gly Leu Phe Lys
130 135 140

Leu Ile Glu Ser Tyr Leu Lys Asp His Pro Asp Ala Tyr Asn
145 150 155

P200101752 sekvensliste.ST25

<210> 3

<211> 159

<212> PRT

<213> Malus domestica

<400> 3

Gly Val Tyr Thr Tyr Glu Asn Glu Tyr Thr Ser Val Ile Pro Pro Ala
1 5 10 15

Arg Leu Phe Lys Ala Phe Val Leu Asp Ala Asp Asn Leu Ile Pro Lys
20 25 30

Ile Ala Pro Gln Ala Ile Lys His Ala Glu Ile Leu Glu Gly Asp Gly
35 40 45

Gly Pro Gly Thr Ile Lys Lys Ile Thr Phe Gly Glu Gly Ser Gln Tyr
50 55 60

Gly Tyr Val Lys His Lys Ile Asp Ser Val Asp Glu Ala Asn Tyr Ser
65 70 75 80

Tyr Ala Tyr Thr Leu Ile Glu Gly Asp Ala Leu Thr Asp Thr Ile Glu
85 90 95

Lys Val Ser Tyr Glu Thr Lys Leu Val Ala Thr Pro Asp Gly Gly Ser
100 105 110

Ile Ile Lys Ser Ile Ser His Tyr His Thr Lys Gly Asp Val Glu Ile
115 120 125

Met Glu Glu His Val Lys Ala Gly Lys Glu Lys Ala His Gly Leu Phe
130 135 140

Lys Leu Ile Glu Ser Tyr Leu Leu Asp His Ser Asp Ala Tyr Asn
145 150 155